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VOLUME 36 AUGUST 1958 NUMBER 8

BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

I. PHYSICAL MEASUREMENTS, PLASMA CHOLESTEROL, AND ELECTROLYTE LEVELS[1]

D. R. IDLER AND H. TSUYUKI

Abstract

Physical measurements have been made on all major organs and tissues of a pure race of migrating adult *Oncorhynchus nerka*. The data show that the population was sufficiently uniform in size that average weights and measurements performed on fish taken at three locations on the migration route quite accurately represented a standard fish of both sexes. The results will enable chemical analyses to be interpreted as changes in absolute amounts of a given tissue component rather than only on a percentage basis. The merits of this approach to an evaluation of changes in migrating salmon is demonstrated by the magnitude of the weight changes in many tissues during the 715-mile migration. A standard male lost only 11% of its body flesh whereas a standard female lost 30%. The alimentary tract decreased to one sixth of the initial weight. The milt increased by 51% while the roe increased by 272%. Thus, it is apparent that percentage composition data cannot show quantitative changes in a fish as it moves up the river. The results are discussed in relation to the findings of others on Salmonidae.

Plasma cholesterol, magnesium, calcium, sulphate, and urea levels were measured on pooled blood samples from the 216 fish.

Introduction

Many of the physiological and biochemical changes occurring during the spawning migration of Salmonidae have been investigated. Interest has been primarily stimulated by the fact that the fish do not feed for many weeks or in some cases months (1, 2, 3, 4, 5). This is also a time of great activity. Tissue energy reserves must be mobilized both for the enlargement of the gonads and for the trip to the spawning grounds, which are often many hundreds of miles from the sea (1, 2, 3, 4).

The expenditure of fat and protein reserves by the Atlantic salmon *Salmo salar* (2, 3) and the Pacific coast *Oncorhynchus tschawytscha* (spring salmon) has been investigated (1). None of these studies appears to provide data which would permit energy expenditures to be calculated either on a time or distance of travel basis. The reasons for this differ in each investigation and include: (1) non-continuous sampling during the spawning migration; (2) too few samples; (3) too few chemical analyses; (4) difficulties in converting

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Contribution from the Fisheries Research Board of Canada, Chemistry Section, Technological Station, Vancouver 2, B.C.

data to a standard fish owing to large variations in the dimensions of individuals. These limitations were overcome to a large degree by the studies of Pentegov *et al.* on migrating *Oncorhynchus keta* (chum salmon) on the Amur River (4). This comprehensive study showed for the first time the energy expenditures of male and female fish per unit distance over the entire freshwater migration of approximately 700 miles. The relatively uniform size and stage of maturity of the population made it possible, within reasonable limits, to accomplish the same results as would be obtained if one could perform a complete proximate analysis on a fish, then return it to the water, and recapture it further upstream. Even this magnificent effort was hampered to some degree by the variations in individual fish which apparently were sometimes not adequately compensated for by the number of samples available. Variations in flesh depot fat in limited flesh segments have been investigated for sockeye salmon (*Oncorhynchus nerka*) on the Columbia River (6). A report of limited scope is available on fat and protein analyses for sockeye on the Harrison River system (7).

In 1956 and again this year, members of the staff of this Station collaborated with the International Pacific Salmon Fisheries Commission biologists on a program designed to study the energy expenditures of Fraser River sockeye migrants to Stuart and Chilco lakes.* The Stuart Lake run has now been investigated for 2 consecutive years. The ability of the biologists to select a pure race of fish of nearly uniform length and weight suggested an excellent opportunity to investigate the more fundamental biochemical changes in individual organs and tissues in a manner analogous to the determination of total energy reserves in the entire fish.

The distribution of flesh nitrogen compounds (8, 9), plasma, and flesh cholesterol, and certain plasma electrolyte levels has been completed. This investigation was carried out on 216 fish representing both sexes of sockeye in three different phases of migration. Proximate analyses, qualitative and quantitative distribution of phosphorus compounds, creatine, inositol, and nucleic acid levels are under investigation by various scientists at this Station. These studies will not be completed for some time.

This initial report provides necessary background information on the fish used in the phases of the project completed to date. It demonstrates also the degree of success achieved in selecting fish of uniform length and weight. There appears to be no available information in the literature for salmon concerning the electrolytes determined in the present study. Blood cholesterol has been measured on an unspecified species of sexually mature salmon taken in a river in Japan (10).

Methods

All fish were caught in reef nets to minimize struggle; they were anaesthetized in ice water before they were bled by cutting off the tail to sever the caudal artery. Heparin was added to the blood, which was centrifuged immediately and the plasma transported to the laboratory in dry ice. Individual

*It is anticipated that the results of the study will be published soon.

fish were placed in polyethylene bags and frozen in brine (−19° C.) at Lummi Island, and on dry ice at the more distant points. The fish were kept at −30° C. until they could be processed within a few days. They were thawed overnight at 0° C. by which time it was just possible to slit the belly wall and remove the frozen viscera. All tissues were kept in polyethylene bags on dry ice until the required number of organs to complete a group was obtained. Four groups of male and four groups of female fish were obtained from each location. The sizes of the groups are shown in Table I.

TABLE I

SAMPLING OF SOCKEYE SALMON

Sex	Group	Number of fish per group		
		Lummi	Lillooet	Forfar
♂	1	7	10	10
♂	2	6	10	5
♂	3	5	10	6
♂	4	5	10	4
♀	1	8	10	14
♀	2	7	10	14
♀	3	9	10	13
♀	4	9	10	14

The internal organs of each group of fish were separated, then pooled and weighed while still frozen. Each eviscerated fish was then weighed both in water and air, as was the tail. Specific gravities were calculated from these data. Each eviscerated fish was then filleted and the entire body flesh portion kept separate from the bone, skin, head, and tail.

Plasma cholesterol was determined by the procedure of Sperry and Webb (11) and sulphate sulphur was determined as described by Cope (12). Well-established procedures were employed to determine calcium as the oxalate, magnesium as magnesium ammonium phosphate, and urea with urease (13).

Results and Discussion

The physical changes in the fish from Lummi Island, where they were in water of low salt content, to Lillooet on the Fraser River and to the spawning grounds at Forfar Creek are shown in Table II. The distances are given in Table IV. Although each fish was measured and its weight recorded the large number of samples necessitates the reporting of averages only for each group. For this reason most of the data have been statistically analyzed. In the discussion "very significant" refers to probability as obtained by the "t" test of .001 or less. When p is between .05 and .001 it is described as significant. When p is greater than .05 no significance is ascribed to the data. All data are recorded as mean ± standard error. Since each group of fish was not exactly the same size, slightly different values were obtained when mean values were calculated on individual fish rather than on group averages. This was particularly true for males at Lummi Island and Forfar Creek. Because of

the complexity of the calculations individual fish were analyzed statistically only for total weight, body length, flesh weight, and weight of the eviscerated fish at Lummi Island. This was done to make the corrections to a standard fish as accurate as possible.

Changes in the Tissues and Organs

A detailed discussion of these changes will be given when proximate analyses are completed. For the present certain observations appear to be pertinent.

It is a well-known fact that the snout length of males increases significantly during migration whereas that of females increases but to a lesser extent. The present study confirms this fact and demonstrates the quantitative aspects for sockeye salmon on a very uniform size population. The male snout increased by 52% and the female by 25% during the migration (Table II). The body length would be expected to remain constant for both sexes with possibly a very small increase during migration owing to the portion of the head which was included in this measurement. The female groups (Table II) show that this constancy has been realized. The average body length of the 23 male fish taken at Lummi Island was somewhat short (49.9 cm.) but the difference was not statistically significant. This situation was anticipated and an additional 17, 35, and 18 male fish were measured at the three consecutive points. When these data were included males averaged 50.2, 50.2, and 50.3 cm. at each consecutive point. All weight data for males at Lummi Island were corrected to body length 50.2 from 49.9 cm. on the previously established basis that the body weight is proportional to the length cubed when the correction is small as it is in this instance (3). There was a significant weight loss in both males and females between Lummi Island and Lillooet. In spite of the extensive depletion of fat and protein stores the males regained their original body weight while the females lost no more on the run to the spawning grounds. Tissue water is the prime factor in explaining this observation but this will be discussed when proximate analyses are completed. The losses in fat and protein with an accompanying percentage increase in body water is well established for migrating salmon (1, 3) and results in the very significant changes in specific gravity of both sexes (Table II). A thickening of the skin during spawning migration has been observed in spring salmon (1, 5). The increase in snout length has already been mentioned. Table III shows that the tail also increased in weight. Since the fish do not feed once they begin the river migration it is not surprising that the viscera of both sexes decreased very significantly in weight. Table III shows that this loss is primarily a reflection of a nearly complete wasting of the alimentary tract. When considering the energy expended during the migration this factor should be taken into consideration. The heart and kidney of both sexes underwent relatively little change in weight. The spleen of both sexes first decreased then increased in weight. The sexes started out very nearly equal in weight when the difference in body length is taken into consideration. However, a comparison of the sexes shows that the females had significantly larger livers than the males. The males had significantly larger alimentary tracts and kidneys.

TABLE II

LENGTH AND WEIGHT CHANGES OF SOCKEYE SALMON DURING SPAWNING MIGRATION

Site	Sex	Groups	Total fish, g.	Total length, cm.	Standard length, A	Snout length, B	Body length, A–B	Evis. fish, g.	Head, skin bones, tail, g.	Flesh, g.	Sp. grounds evis. fish
Lummi Is.	♂	4	2565±42	59.3	53.6	3.4±.11	50.2±.29	2322±40	881±33	1467±21	1.033±.000
Lillooet	♂	4	2371±26	59.3	54.1	3.9±.15	50.2±.00	2185±25	871±10	1302±51	1.024±.000
Forfar Cr.	♂	4	2583±51	61.1	55.5	5.2± 16	50.3±.28	2382±47	1057±10	1304±40	1.012±.000
Lummi Is	♀	4	2376±26	57.4	52.0	2.8±.04	49.2±.29	2151±19	785±15	1335±13	1.037±.000
Lillooet	♀	4	2156± 4	57.6	52.4	3.1±.05	49.3±.40	1908±41	765±20	1135±30	1.024±.000
Forfar Cr.	♀	4	2168±10	58.2	52.8	3.5±.04	49.3±.26	1775±10	834±12	934±10	1.015±.000

TABLE III

WEIGHT AND LENGTH CHANGES IN THE ORGANS OF SOCKEYE SALMON DURING SPAWNING MIGRATION

Site	Sex	Groups	Heart, g.	Liver, g.	Spleen, g.	Kidney, g.	Alimentary tract, g.	Viscera, g.	Gonads, g.	Tail, g.
Lummi Is.	♂	4	6.10±.43	31.6±0.8	2.90±.26	26.0±0.8	90.5±3.3	157±5.0	56.5±2.3	64. ±2.3
Lillooet	♂	4	5.88±.26	25.4±0.6	1.98±.05	23.3±0.7	30.1±0.4	86.7±1.7	82.0±2.8	58. ±0.5
Forfar Cr.	♂	4	5.80±.28	37.6±1.0	3.60±.23	28.1±1.0	16.4±1.0	91.5±2.4	85.4±2.4	82. ±3.1
Lummi Is.	♀	4	5.05±.13	40.2±0.5	2.04±.09	21.6±0.7	78.3±1.6	147±3.0	80.1±0.8	59. ±0.6
Lillooet	♀	4	4.95±.21	44.6±0.9	1.50±.08	19.9±0.2	26.2±0.8	97.2±3.6	138±3.7	52. ±1.5
Forfar Cr.	♀	4	4.58±.06	40.5±1.6	2.17±.18	19.7±0.2	12.9±2.7	79.9±2.1	298±2.3	77. ±0.9

Gonads

The energy which a starving fish must expend to produce milt or roe has been emphasized for *Salmo salar* in the rivers of Scotland (3). In May and June the ovaries represented only 1.2% of the total weight of the fish, whereas in September and October on the upper river the ovaries were 23.2% of the fish. The testes increased from 0.15% to 3.32% of the weight of the fish. Greene's data for Columbia River *O. tschawytscha* shows that the ovaries represented 5% (two fish) of the weight of the fish at the mouth of the river and 20% (four fish) on the spawning grounds but the number of fish was limited and the fish were quite variable in size. Miescher-Rüsch emphasized that abnormal proportions were exhibited between the sexual glands and the weight of the body for extreme sizes of Atlantic salmon (2). He concluded that the large majority of Rhine salmon did not develop significant milt or roe while in the sea. This is not a general phenomenon, however, as Paton has shown that both male and female Atlantic salmon entering rivers in Scotland showed large increases in milt and roe from May through October. For the ovaries this variation was from 1.2% in May and June to 12.4% in September and October. In the same time period the milt of incoming migrants increased from 0.15% to 2.71% (3).

In comparing the data for *O. nerka* with that outlined for other Salmonidae certain points are worthy of mention. The Atlantic salmon, *S. salar*, is generally a much larger fish. Thus the fish in the present study averaged about 2.5 kg., whereas the Rhine salmon examined at Basel averaged 8 kg. The *O. tschawytscha* on which Greene carried out his studies varied from about 7.5 to 16 kg. *O. keta* investigated by Pentegov averaged around 4 kg. The Atlantic salmon frequently return to the sea after spawning whereas *Oncorhynchus* do not. The Rhine salmon spend many months in the spawning area waiting for the gonads to ripen, whereas in the present study only a few days separated arrival and spawning. However, both genera cease to take food by the time the fish reach fresh water (and probably before) and then begin an extended period of starvation at a time when the energy demands both for swimming and sexual maturation are extensive.

The study by Pentegov *et al.* on migrating chum salmon (*O. keta*) on the Amur River most closely parallels the present study (4). The total distance of the migration, somewhat over 700 miles, was very comparable (Table IV). The water temperatures were similar. The distance to an intermediate point on the migration was nearly identical. Chum salmon migrate somewhat slower than sockeye salmon. The gonads of both the male sockeye and chum salmon did not increase in weight after the first 250 miles of river migration (Tables III and IV). The gonads of both male and female chum salmon on the Amur River were heavier than those of sockeye salmon in relation to body weight on entering the river. The milt of both species appeared to decrease in relation to body weight in the later stages of migration (Table IV). For sockeye this change was not significant.

The energy expenditure to produce roe by both female sockeye and chum salmon was considerably greater than the expenditure by males to produce milt. Of particular importance was the relatively small weight of milt which must be produced by males during the starvation period in the river in comparison to the roe produced by the female (Table III).

TABLE IV

COMPARISON OF OVARIES FOR STUART LAKE SOCKEYE AND AMUR RIVER CHUM SALMON

Location	Days	Miles	H_2O temp., °F.	Milt, %*	Roe, %*
Fraser River					
Lummi Is.	0	0	62 (est.)	2.20	3.37
Lillooet	10	250	63	3.46	6.40
Sp. grounds	27	715	48	3.31	13.7
Amur River					
Langre Is.	0	0	63	4.11	7.02
Sophiskoya	12	253	62	4.21	10.1
Sp. grounds	33	740	48	3.08	17.4

*Expressed as percentage of the live weight of the standard fish at each station.

Plasma Cholesterol and Electrolytes

The levels of free and esterified cholesterol found in male and female plasma are given in Table V. The "normal" total cholesterol levels at the beginning of the upriver migration were very high (570 mg.%) compared to warm-blooded animals (150–190 mg.%) (13). However, average levels of 662 mg. % have been reported for carp blood (14). The decrease in total plasma cholesterol from Lummi Island to the spawning grounds (715 miles) was far greater in female (65%) than in male fish (31%). Females also lost more free cholesterol (64%) than did males (53%). Over the relatively short distance between Lummi Island and Lillooet (250 miles) the females showed no decrease in free cholesterol and a 13% decrease in total cholesterol. The males over the same distance lost 23% total cholesterol and 35% free cholesterol. It can thus be concluded that the females began to lose plasma cholesterol later in the migration than did the males but the losses in the later stages were much greater in the female. The ratio of free to total cholesterol steadily decreased as the males moved up the river. The ratio was 0.46 at Lummi Island, 0.39 at Lillooet, and 0.34 at the spawning grounds. The females show less variation in the ratio of free to total cholesterol. The ratio was 0.38 at Lummi Island and Forfar Creek and 0.44 at Lillooet. On arrival of the fish at the spawning grounds, which for this race occurs only a few days before spawning, both the total and free cholesterol levels were significantly higher in the male. However, for an unspecified species of sexually mature salmon (probably *O. keta*) in a Japanese river, similar levels of both free (75–80 mg.%) and total cholesterol (150 mg.%) have been reported for both sexes (10). The present

TABLE V

PLASMA CHOLESTEROL AND ELECTROLYTE LEVELS IN MIGRATING SOCKEYE SALMON

Components, mg.%	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Free cholesterol	♂	261 ± 18(23)*	170 ± 17(40)* .02†	134 ± 15(25)* .1†
	♀	215 ± 9(33)*	220 ± 24(40)* .8†	77 ± 6(55)* .001†
Total cholesterol	♂	570 ± 19	436 ± 38 .02	394 ± 25 .4
	♀	572 ± 19	499 ± 24 .05	202 ± 20 .01
Calcium	♂	21.6 ± 0.6	19.9 ± 1.3 .2	12.5 ± 0.8 .001
	♀	27.3 ± 0.4	23.8 ± 1.1 .02	15.5 ± 0.6 .001
Magnesium	♂	1.58 ± 0.16	3.71 ± 0.25 .001	2.15 ± 0.11 .01
	♀	0.86 ± 0.06	2.88 ± 0.18 .001	2.18 ± 0.12 .01
Urea N	♂	6.0 ± 0.3	4.3 ± 0.7 .05	4.9 ± 0.2 .3
	♀	6.2 ± 0.6	3.7 ± 0.5 .02	4.2 ± 0.4 .4
Inorganic sulphate as S	♂	12.5 ± 0.2	5.5 ± 0.3 .001	9.6 ± 0.3 .001
	♀	13.0 ± 0.7	3.9 ± 0.3 .001	9.8 ± 0.2 .001

*Total number of fish divided into four groups.
†Significance of difference from value immediately to left.

study has shown that the plasma cholesterol levels were extremely high in sexually immature sockeye salmon and decreased with increasing sexual maturity and/or decreasing energy reserves.

The carbohydrate and glycogen content of salmon liver and flesh is known to be small (15). However, it was considered of interest to determine levels of plasma magnesium. The results (Table V) show that they were generally within the range of 1–3 mg.% reported for human serum (13). Carp serum is reported to contain 3.32 mg.% (14). The very significant increase during the migration may reflect an increased carbohydrate metabolism during this very active phase of the salmon's life, but this is purely conjecture. The recent isolation of a new sulphate-containing nucleotide from salmon liver in this laboratory prompted the determination of plasma sulphate (16). The levels (Table V) at all stages of migration were very significantly higher than those reported for human serum (0.9–1.1 mg.%) (13) or carp serum (0.94 mg.%) (14). The difference between the initial and final levels can probably be accounted for by blood dilution, which is reported to be about 12% for *O. tschawytscha* (1). The significant drop during the early phase of migration

and the subsequent rise in the later phase is analogous to the change reported in human pregnancy (17). Urea nitrogen levels, 6 mg.% for males and 6.2 mg.% for females, were lower than those normally reported for humans (10–15 mg.%) (13) but somewhat higher than for fresh-water carp (3.5 mg.%) and trout (2.6 mg.%) (14). The changes during spawning migration can probably be largely accounted for by dilution of the blood. The calcium levels, 21.6 and 27.3 mg.%, respectively, for males and females, were much higher than the 9–11.5 mg.% reported for man (13) or the 11.5 mg.% reported for carp serum (14). The calcium levels like the cholesterol levels decreased in the final stage of sexual maturity to the same magnitude as is found in humans. Parathyroid-like function has been attributed to the ultimobranchial gland of fish (18, 19, 20). However, too little is known of the functions of this gland at the present time to justify more than a suggestion that decreased activity of the gland may be associated with the continually decreasing blood calcium levels. By analogy with mammalian serum the decreased calcium levels should be accompanied by a decrease in serum protein (21, 22). This will be investigated.

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BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

II. CHOLESTEROL, FAT, PROTEIN, AND WATER IN THE FLESH OF STANDARD FISH[1]

D. R. IDLER AND I. BITNERS

Abstract

The absolute changes in free and esterified cholesterol, fat, protein, and water are discussed for the flesh of standard male and female salmon during the spawning migration. The data were obtained for 12 groups of male and 12 groups of female sockeye representing 216 fish. Fish were caught at three locations: below the entrance to the river, 250 miles up the river, and at the spawning grounds, which were 715 miles from the first sampling station.

Introduction

Recently, a pure race of Fraser River sockeye salmon was sampled at the beginning, at an intermediate point, and at the end of a 715-mile spawning migration (1). The data showed that the large number of fish taken (216) and the uniformity of the population permitted an accurate standard fish to be calculated for each sex. In effect, the realization of a standard fish permits one to obtain the data which would be obtained if a fish could be analyzed, then returned to the water and recaptured at a later time. By this technique not only percentage changes but absolute weight changes in tissue components may be determined. This report will consider the absolute weight changes in the flesh fat, protein, water, and cholesterol levels in standard male and female sockeye salmon as they migrate 250 miles from Lummi Island to Lillooet and then a further 460 miles to the spawning grounds at Forfar Creek.

Methods

The sockeye salmon were caught in reef nets and transported to the laboratory at the temperature of dry ice as previously described (1). The fish were thawed overnight and the frozen viscera including the kidneys were removed. The head and tail were severed and the well-scraped skin and bones were separated from the body flesh. The flesh of males and females was separated and divided into groups as previously described (1). The frozen flesh was put through an electric cutter and the entire sample (15 to 42 lb.) was thoroughly mixed, and representative 1-lb. samples sealed in enamelled cans and either stored at $-30°$ C. or heat processed. The heat-processed samples were employed for the analyses described in this report.

Fat was determined by a rapid method developed specifically for canned salmon (2). Water was determined with an automatic infrared moisture balance. The fat was then taken up in acetone:ethanol, 1:1 and free and esterified cholesterol determined by the Sperry–Webb procedure (3).

[1]Manuscript received April 9, 1958.
Contribution from the Fisheries Research Board of Canada, Chemistry Section, Technological Station, Vancouver 2, B.C.

Results and Discussion

The proximate analyses for the male and female fish at each location are shown in Table I. The original moisture content and the percentage increase in moisture in the flesh was essentially the same for both sexes. The females began the spawning migration at Lummi Island with 11% more fat than the males and up to Lillooet (250 miles) both sexes appear to have lost about the same percentage of the initial fat content resulting in a 16% greater loss for the female because of the higher fat content at Lummi Island. Both sexes began the migration with the same percentage of protein and appeared to lose the same percentage in the first 250 miles. Over the entire migration distance the females used approximately 18% more protein than the males. *The percentage composition data do not show the true picture for the fat and protein consumption of the two sexes because they make no allowance for changes in the total weight of the flesh as the fish move up the river.* This point is demonstrated in Table II of the preceding paper (1) where it is shown that the body flesh of a standard male sockeye salmon decreases from 1467 g. at Lummi Island to 1300 g. at Lillooet and Forfar Creek. The flesh of the standard female sockeye weighed 1335 g. at Lummi Island, 1135 g. at Lillooet, and only 934 g. at Forfar Creek. Table II shows the actual fat, protein, and water in the flesh of an average standard male and female salmon on a weight basis at each of the three locations. Table III shows these data in a more easily interpreted form based on the changes in total weight of fat and protein per unit distance.

TABLE I

PROXIMATE ANALYSES AND CHOLESTEROL CONTENT OF THE FLESH OF MIGRATING SOCKEYE SALMON*

Components	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Moisture, %	♂	67.2±0.11(23)†	70.7±0.23(40)†	78.2±0.23(25)†
	♀	67.0±0.16(33)†	71.0±0.12(40)†	78.0±0.07(55)†
Fat, %	♂	9.34±0.24	5.07±0.24	3.23±0.23
	♀	10.55±0.12	5.59±0.19	2.70±0.11
Protein N,‡ %	♂	3.508±0.047	3.283±0.036	2.684±0.039
	♀	3.518±0.107	3.385±0.061	2.835±0.020
Moisture+fat+protein, %	♂	98.47	96.29	98.21
	♀	99.53	97.75	98.42
Free cholesterol, mg./100 g.	♂	25.7±0.81	30.9±0.22 0.01§	31.1±0.40 0.8§
	♀	25.6±0.24	30.6±0.22 0.001§	34.7±0.77 0.001§
Total cholesterol	♂	29.2±0.68	34.1±0.27 0.01	36.7±0.77 0.05
	♀	29.0±0.48	32.6±0.24 0.01	35.9±0.40 0.01

*± Standard errors.
†Total number of fish divided into four groups.
‡Total flesh protein nitrogen was determined in connection with a study on the distribution of nitrogenous components of flesh (6).
§Significance of difference from value immediately to left.

TABLE II

CHANGES IN FAT, PROTEIN, WATER, AND CHOLESTEROL IN THE FLESH OF MIGRATING SOCKEYE SALMON OF STANDARD LENGTH

Components, g. in flesh	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Moisture	♂	986	921	1020
	♀	894	806	729
Fat	♂	137	66	42
	♀	141	64	25
Protein	♂	322	267	219
	♀	294	240	165
Free cholesterol	♂	0.377	0.402	0.406
	♀	0.342	0.347	0.324
Total cholesterol	♂	0.428	0.444	0.479
	♀	0.387	0.370	0.335

TABLE III

EXPENDITURE OF FLESH FAT AND PROTEIN BY MIGRATING SOCKEYE SALMON OF STANDARD LENGTH

Location	Sex	Component expended by standard fish in mg./mile	
		Fat	Protein
Lummi Island to Lillooet	♂	284	220
	♀	304	216
Lillooet to Forfar Creek	♂	52	104
	♀	85	163
Lummi Island to Forfar Creek	♂	132	144
	♀	161	180

The changes in fat for the standard fish are quite different than when considered on a percentage basis. The standard females use only 7% more fat than the standard males over the first 250 miles and they use an almost identical weight of protein. Since the initial weight at Lummi Island of the standard female fish (2376 g.) is 92.5% of the initial weight of the standard male fish (2565 g.) the female expends 15.3% more flesh fat and 6.5% more flesh protein than a male fish of the same body weight. The most significant difference in male and female fish over the 250 miles from Lummi Island to Lillooet is the change in the weight of gonads. The male milt increases by only 25.5 g., whereas the female roe increases by 57.9 g. (1). The female would therefore be expected to consume more body fat and protein if the energy expenditure for swimming was the same for both sexes. The data appear to substantiate this supposition. During the final 460 miles the standard females use 57% more flesh protein and 63% more flesh fat per unit distance than does the

standard male. The standard male at Lillooet weighed 2371 g. and 2583 g. at the spawning grounds, whereas the standard female weighed 2156 g. at Lillooet and 2168 g. at the spawning grounds (1). When the average weight of the standard fish for each sex is considered over this 460 miles the standard female expends 87.2% more flesh fat and 80.6% more flesh protein than does a standard male of the same body weight. The most significant difference in the two sexes over the final 460 miles of the spawning migration is in the weight of the gonads. The male milt increases by only 3 g., whereas the female roe increases by 160 g. An approximate calculation based on 17.6% protein and 9.2% fat for the ovaries (4) shows that the 157-g. excess of gonad produced by the female as compared to the male represents approximately 27.6 g. of protein and 14.5 g. of fat. Table II shows that the standard female expends 15 g. more fat and 27 g. more protein than the standard male over the last 460 miles. This excellent agreement appears to offer convincing evidence that the standard female and male sockeye salmon expend nearly identical energy for swimming and the excess flesh fat and protein consumed by the female can be attributed to the larger weight of the gonads. The standard female expends 22% more flesh fat and 25% more flesh protein than does the standard male over the 715-mile migration route (Table III). The average weight of a standard male fish from Lummi Island to the spawning grounds is 2574 g. as compared to 2272 g. for the female (1). When this weight difference is taken into account the standard female expends 38% more flesh fat and 42% more flesh protein than a standard male of the same weight. On the same equal weight basis the standard female begins the migration at Lummi Island with 10.2% more fat and 1.2% less protein. The demands on flesh fat and protein are considerably greater for the female than for the male and only in the case of fat are somewhat greater reserves available.

For the first 250 miles the standard males expend 29% more fat than protein and the females expend 40.5% more fat. For the last 460 miles the utilization reverses and the standard male expends 100% more protein than fat while the standard female expends 92% more protein (Table III).

Paton, in referring to water changes in the flesh of migrating *Salmo salar* states that "it is this increase in the percentage water of the flesh which maintains the weight of the fish per fish of standard length, although the solids as a whole have diminished" (5). This thought is prevalent in the literature in the more restricted sense that the percentage of fat plus water is a constant. There is no doubt that the percentage of water does increase as the percentage of fat decreases but the statement quoted above is not valid for the present study. The flesh of neither sex takes on water in sufficient quantity to replace depleted fat and protein reserves. Table I shows that even on a percentage basis the fat plus water at different points for the same sex are not very constant. Over the 715-mile route the flesh of the standard female lost 129 g. of protein, 116 g. of fat, and lost (not gained) 165 g. of water (Table II). The flesh of the standard males lost 103 g. of protein, 95 g. of fat, and only gained 34 g. of water, resulting in a loss in the weight of the flesh of 164 g. as compared

to 410 g. for the standard female. Table I shows that, in general, fat, protein, and water total nearly 100%. However, the data for both sexes strongly suggest that another flesh component (or components) is more significant at Lillooet than at Lummi Island or Forfar Creek. In the present study the standard eviscerated male fish which weighs 2322 g. at Lummi Island and 2382 g. at Forfar Creek does confirm Paton's statement that the weight of the fish remains essentially constant but the constancy is due only partly to the increased water in the flesh but primarily to gain in weight of the head, skin, bones, and tail from 881 g. to 1057 g. (1). The head, skin, bones, and tail of the female increases from 785 g. at Lummi Island to 834 g. at Forfar Creek, but the magnitude is not sufficient to maintain the weight of the standard eviscerated female which drops from 2151 g. at Lummi Island to 1775 g. at Forfar Creek (1).

Both the male and female standard fish showed a loss of water for the first 250 miles of the spawning migration. The loss was 6.6% for the males and 9.8% for the females. Over the last 460 miles the males gained 10.7% water while the females lost 9.7%.

Table I shows the changes in free and total cholesterol expressed in mg./100 g. of flesh. The free cholesterol per unit weight of flesh of females increased 19.5% in the first 250 miles and a further 13.5% in the last 460 miles for an over-all increase of 35.6% during the migration. However, when the loss in weight of the total flesh of female standard fish is considered (1) the total free cholesterol of the flesh increases only insignificantly (1.5%) (Table II) in the first 250 miles and decreased 6.5% over the last 460 miles for an over-all loss of 5.3% during the migration. The males showed an increase of 20% in free cholesterol per unit weight of flesh in the first 250 miles and no change after this point. The flesh of the standard male decreases from 1467 g. to 1302 g. over the first 250 miles and as a result the increase in free cholesterol per standard fish is only 6.6% . The weight of the male flesh does not change during the last 460 miles, neither does the percentage cholesterol.

The total cholesterol increased steadily for both sexes over the migration route when the results are considered per unit weight of flesh (Table I). However, when changes in flesh weight are taken into consideration the standard male fish showed a gain in total cholesterol of 12% and the standard female a loss of 13% over the 715-mile migration.

The esterified cholesterol represents only 12% of the total at Lummi Island for the females and this decreased to 6.1% at Lillooet and 3.3% at Forfar Creek. The male flesh had 12% of the total cholesterol in an esterified form at Lummi Island, 9.4% at Lillooet, and 15% at Forfar Creek.

The preceding paper showed the very considerable decreases in plasma cholesterol for both sexes over the migration route (1). It seems surprising that while flesh reserves of fat and protein and plasma cholesterol were so extensively depleted the cholesterol content of the flesh of both sexes actually increased per unit weight of tissue. In the case of the male even the standard fish showed an increase in flesh cholesterol while the standard female suffered

no great loss. It should be emphasized, however, that whereas the flesh of a standard male fish contained 322 g. of protein and 137 g. of fat at Lummi Island, it only contained 428 mg. of cholesterol (Table II). Thus, from an energy consideration, it makes little difference whether the fish does or does not utilize the flesh cholesterol.

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BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

III. CHANGES IN THE PROTEIN AND NON-PROTEIN NITROGEN FRACTIONS IN MUSCLES OF MIGRATING SOCKEYE SALMON[1]

D. W. DUNCAN AND H. L. A. TARR

Abstract

The nitrogen content of three protein fractions and one non-protein nitrogen fraction of muscles of migrating sockeye salmon was determined at three points on the migratory route. The results have been expressed as milligrams of nitrogen per 100 g. of muscle, as per cent of the total muscle nitrogen, and as grams of nitrogen per average (standard) male or female fish.

Introduction

The only previous studies in which determinations of changes in total protein, water-soluble nitrogenous constituents, and lipids in muscles of migrating salmon were made are those of C. W. Greene (1) and C. H. Greene (2) with "spring salmon" (*Oncorhynchus tschawytscha*). They studied only a single protein fraction which was prepared by heat coagulation and solvent extraction of the whole muscles. It was found that the protein content of the wet muscle tissue decreased from 20.07 to 14.02%, and that the water content increased from 74.8 to 81.5% as the fish migrated from the river mouth to the spawning grounds (1). The total water-soluble extractive nitrogen increased slightly, the amino nitrogen content increased markedly, and the creatine nitrogen remained practically unchanged during migration (2). In the present investigation changes in three protein fractions and in the non-protein nitrogen of the muscles of migrating sockeye salmon (*Oncorhynchus nerka*) have been determined.

Materials and Methods

The methods of securing representative samples of fish at three points on the migratory route and of preparing the muscle fraction from these have been described in detail (3).

The protein fractionation procedure was based on that which has, with various modifications, been used to prepare protein fractions from mammalian or fish skeletal muscles for many years (4). It involved preparation of four general fractions, namely (1) proteins soluble in cold, approximately neutral salt solution of comparatively high ionic strength and insoluble in salt solutions of low ionic strength (largely actomyosin); (2) proteins soluble in cold neutral salt solutions of low ionic strength ("myogens", "myoalbumens", etc.); (3) proteins insoluble in the above solutions (collagen and elastin; denatured myosin); and (4) nitrogenous compounds not precipitated by cold 5% trichloroacetic acid solution (non-protein nitrogen).

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TABLE I

NITROGEN PARTITION IN PROTEIN AND NON-PROTEIN NITROGEN FRACTIONS OF MUSCLE TISSUE OF MIGRATING SOCKEYE SALMON

Station	No. and sex of fish per group		Mg. of N per g. (wet weight) and % of total N					
			Whole muscle	Stroma	High ionic strength	Low ionic strength	Non-protein	% recovery‡
Lummi Island	7 ♂	mg.	34.64	4.96	15.70	8.77	4.55	
		%	100	14.32	45.32	25.32	13.13	
	6 ♂	mg.	35.28	6.30	15.09	7.94	4.75	
		%	100	17.86	42.77	22.51	13.46	
	5 ♂	mg.	34.20	6.02	15.60	7.45	3.87	
		%	100	17.60	45.61	21.78	11.31	
	5 ♂	mg.	36.20	7.56	17.32	7.68	4.86	
		%	100	22.05	47.84	21.21	13.42	
	Av.	mg.	35.08±0.47	6.21±0.53	15.93±0.58	7.96±0.287	4.43±0.223	
		%	100	17.96±1.66	45.39±1.04	22.69±0.825	12.83±0.51	98.43
	8 ♀	mg.	32.00	8.40	12.40	7.47	4.83	
		%	100	26.25	38.75	23.34	15.09	
	7 ♀	mg.	36.60	7.00	15.81	7.81	4.07	
		%	100	18.13	40.96	18.94	10.54	
	9 ♀	mg.	36.20	7.72	14.90	8.16	4.40	
		%	100	21.33	41.16	22.54	12.15	
	9 ♀	mg.	35.90	5.68	16.68	8.82	4.64	
		%	100	15.82	46.46	24.57	12.92	
	Av.	mg.	35.18±1.07	7.20±0.68	14.95±0.15	7.94±0.288	4.48±0.165	
		%	100	20.55±2.26	42.60±1.64	22.60±1.21	12.61±0.945	98.36
	10 ♂	mg.	33.71	4.50	15.39	9.30	4.58	
		%	100	13.35	45.65	27.59	13.59	
	10 ♂	mg.	32.27	4.42	14.47	8.82	4.70	
		%	100	13.70	44.84	27.33	14.56	
	10 ♂	mg.	33.15	5.36	14.58	9.94	4.71	
		%	100	16.17	43.98	29.28	14.21	
	10 ♂	mg.	32.20	5.25	14.46	9.13	4.74	
		%	100	16.30	44.70	28.35	14.72	
	Av.	mg.	32.83±0.36*	4.88±0.44	14.73±0.20	9.30±0.226†	4.68±0.041	
		%	100	14.86±0.785	44.80±0.345	28.33±0.44†	14.25±0.25†	102.24

Lillooet								
	10 ♀	mg.	34.72	5.44	15.30	9.63	4.60	
		%	100	15.67	44.07	27.73	13.25	
	10 ♀	mg.	34.71	4.95	14.11	9.16	4.91	
		%	100	14.26	40.65	26.39	14.15	
	10 ♀	mg.	32.12	4.97	14.42	9.16	4.56	
		%	100	15.47	44.90	28.52	14.20	
	10 ♀	mg.	33.87	5.27	14.23	9.25	4.37	
		%	100	15.56	42.01	27.31	12.90	
	Av.	mg.	33.85±0.61†	5.16±0.48†	14.52±0.92	9.30±0.111†	4.61±0.111	
		%	100	15.24±0.328	42.91±0.965	27.47±0.44†	13.62±0.323	99.24
	10 ♂	mg.	27.65	4.18	13.05	6.41	4.25	
		%	100	15.12	47.20	23.18	15.37	
	5 ♂	mg.	26.83	4.07	13.30	6.41	4.26	
		%	100	15.11	49.57	23.89	15.88	
	6 ♂	mg.	27.11	4.14	13.39	6.49	4.02	
		%	100	15.27	49.39	23.94	14.83	
	4 ♂	mg.	25.78		13.22	6.33	3.88	
		%	100		51.28	24.55	15.05	
	Av.	mg.	26.84±0.39*	4.13±0.23	13.24±0.27*	6.41±0.240*	4.10±0.091	
		%	100	15.30	49.33±0.84	23.88±0.28*	15.28±0.23†	103.69
Forfar								
	14 ♀	mg.	27.86	3.46	13.49	6.33	4.83	
		%	100	12.42	48.42	22.72	17.34	
	14 ♀	mg.	28.20	4.32	13.81	6.73	4.51	
		%	100	15.32	48.97	23.86	15.99	
	13 ♀	mg.	28.58	4.31	12.86	6.99	4.80	
		%	100	15.08	45.00	24.42	16.79	
	14 ♀	mg.	28.78	4.06	12.27	6.88	4.37	
		%	100	14.11	42.63	23.90	15.18	
	Av.	mg.	28.35±0.20*	4.04±0.086†	13.11±0.34	6.74±0.250*	4.63±0.224	
		%	100	14.25±0.655	46.24±1.50	23.75±0.357*	16.33±0.47†	100.57

*Significance of difference from the corresponding preceding average value = $>t$ 0.01.
†Significance of difference from the corresponding preceding average value = $>t$ 0.05.
‡Average nitrogen recovered in the four fractions as per cent of total nitrogen.
NOTE: Means are tabulated ± standard error.

Salmon muscle (20 g.) was blended in a tightly closed, completely filled, ice-packed stainless steel jar of a Servall Omni-Mixer with 380 ml. of buffered 0.85 *M* NaCl solution (5). An aliquot (250 ml.) of the suspension was centrifuged 30 minutes at 20,000 *g* and 0° C. The supernatant was decanted and the residue (stroma proteins and denatured myosin) dissolved in 25 ml. of 1 *N* NaOH. After storage overnight at 10° C., the solution was adjusted to 50 ml. and 5-ml. aliquots were used for nitrogen determinations. The supernatant liquid was adjusted to 250 ml., and a 100-ml. aliquot was mixed with 900 ml. of water at 0° C. and, after 16–18 hours at 0° C., 350 ml. of the suspension was centrifuged at 20,000 *g* and 0° C. The centrifugate was dissolved in 25 ml. of 1 *N* NaOH and adjusted to 50 ml. with water for nitrogen determinations, using 10-ml. aliquots (high ionic strength fraction). The supernatant liquid was readjusted to 350 ml. with water, and to a 100-ml. aliquot 5 g. of trichloroacetic acid was added with stirring at 0° C. The solution was centrifuged 30 minutes at 20,000 *g* and 0° C. The volume of the supernatant solution was determined, and duplicate 25-ml. aliquots were used for nitrogen determinations (non-protein nitrogen fraction). The trichloroacetic acid precipitate was dissolved in 12 ml. of 1 *N* NaOH, the solution adjusted to 25 ml., and its nitrogen content determined using 10-ml. aliquots (low ionic strength fraction).

Nitrogen was determined by the modification of the Kjeldahl procedure described by Campbell and Hanna (6).

Results

The conditions under which the fish were obtained and the samples were prepared, which involved slow freezing, and thawing, and mincing the flesh in a "silent cutter" (3) undoubtedly caused considerable denaturation of the myosin. The large number of fish sampled, and the transportation distances involved, made it virtually impossible to carry out the fractionation procedure under conditions which would preclude myosin denaturation. In the procedure used denatured myosin would be found in the stroma fraction, and that this is the case is borne out by the finding that the stroma protein fraction accounted for from 14.25 to 20.55% of the total muscle nitrogen, values greatly in excess of the values (3 to 4%) which are considered normal for teleost fish (7). If it is assumed that true stroma proteins account for 3% of the total muscle nitrogen, and that the remaining nitrogen is denatured myosin, then the "high ionic strength" fraction would account for 54.15 to 61.6% of the total nitrogen instead of the 42.6 to 49.33% actually found. The higher values are similar to those reported for fish actomyosin in the literature (4, 8).

When calculated on the basis of milligrams of nitrogen per gram of muscle (per cent basis) (Table I) there is a significant decrease in the total nitrogen content of the fish as they migrate. This decrease is largely due to decreases in the nitrogen content of the high ionic strength plus stroma protein fractions. The concentration of nitrogen in the low ionic strength fraction increased significantly between Lummi Island and Lillooet, and decreased significantly

TABLE II

NITROGEN CONTENT OF MUSCLES OF MIGRATING SOCKEYE SALMON (GRAMS PER STANDARD FISH)

Fraction	Males			Females		
	Lummi Island	Lillooet	Forfar	Lummi Island	Lillooet	Forfar
Whole muscle	51.462	42.754	34.951	46.966	38.376	26.717
Stroma+high ionic strength	32.479	25.540	22.038	29.470	22.329	16.149
Low ionic strength	11.677	12.111	8.354	10.769	10.554	6.347
Non-protein nitrogen	6.499	6.097	5.340	5.988	5.225	4.362

between Lillooet and Forfar. The significant increase in the percentage of nitrogen in the non-protein fraction is due to the significant decrease in the total nitrogen of the samples. The actual composition of the fraction itself in terms of milligrams of nitrogen remained constant within limits of error, and this is verified by the detailed analysis of this fraction (9).

The amount of nitrogen in each of the four fractions when calculated on the basis of the weight of a "standard" fish (3) is recorded in Table II. The values given in this table were obtained by multiplying the average nitrogen concentrations shown in Table I by the weights of standard male or female fish. The marked decrease in the nitrogen of the whole muscle is due to both the marked decrease in weight of the fish and to the lower per cent nitrogen content of the muscle as migration proceeds. The sum of the nitrogen of the high ionic strength and stroma protein fractions also decreases but the decrease is much more pronounced for females than males during the later stages of migration. Initially there is practically no change in the amount of nitrogen available to the fish in the low ionic strength fraction but a sharp decrease occurs during the later stage of migration. The decrease in non-protein nitrogen is entirely due to decrease in weight of the fish and not to the nitrogen concentration of the fraction.

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SMOOTH MUSCLE ELECTROLYTES[1]

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With the technical assistance of KATHLEEN ROBINSON

Abstract

The sodium, potassium, chloride, and water concentrations have been analyzed in relatively pure smooth muscles of electrophysiological interest and compared to similar analyses of other muscle types. The smooth muscles analyzed included isolated longitudinal myometrium from the uteri of various species and the taenia coli from guinea pigs and rabbits. The lack of accurate measurements of extracellular fluid volume was stressed and various assumptions were made to provide limiting values for this volume. Within these limits, various distributions of the ions studied were calculated. The outstanding feature of the ion distribution was the occurrence of an apparently hyperosmotic concentration of monovalent cation (Na+K) irrespective of its assumed volume of distribution. Evidence was discussed suggesting that this finding should be attributed to cation binding and/or large quantities of polyvalent intracellular anion. Evidence was presented suggesting active potassium uptake and active extrusion of sodium as the mechanisms of distribution of these ions. Chloride distribution was compatible with the assumption of distribution according to its equilibrium potential.

Introduction

The electrolyte concentrations in smooth muscle cells are of interest because the transmembrane resting and action potentials observed in this tissue differ from those occurring in either skeletal or cardiac muscle both in their magnitude (1–7) and probably in the mechanism of their production. Resting membrane potentials average only about 50 mv. in various types of smooth muscle (7, 8) and even when immersed in isotonic solutions of potassium salts, transmembrane potentials of 30–35 mv. have been recorded (7). Action potentials of smooth muscle also are relatively small (5–60 mv.) (2, 4, 5) and are less dependent, in amplitude and rate of depolarization, upon the external sodium concentration (5, 7).

Previous work from this laboratory (9) has indicated that stomach muscle may differ in electrolyte composition from other types of muscle. Specifically the chloride space was found to be larger than the sodium space. It follows that in this tissue either chloride is bound in a non-ionic form by some tissue component, or it is present in appreciable concentrations (greater than sodium) within the interior of cells. Furthermore, stomach muscle and striated muscle did not respond to controlled alterations in extracellular electrolyte concentrations in the same manner.

More extensive data on the electrolyte composition of smooth muscles (such as uterus and taenia coli) whose transmembrane potentials have been examined are needed for an adequate interpretation of the ionic mechanisms underlying these electrical phenomena.

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Methods

Preparation of Animals

Hormonal Treatment

Estrogen.—Immature rabbits (2–3 lb.) and cats (2 lb.) were treated with diethylstilbestrol (100 μg. per day for 7 days). Injections were made subcutaneously.

Estrogen plus progesterone.—Estrogen-treated animals were continued on estrogen for 3 additional days and progesterone (2 mg./day) was given at a separate injection site.

Removal of Tissues

Rodents were killed by a blow at the foramen magnum and tissues excised rapidly for analyses. Tissues were removed from cats under pentobarbital anesthesia (40 mg./kg., intraperitoneal). Human tissues were removed during Caesarean sections or during hysterectomy. They were weighed immediately in the operating room. All tissues were blotted as free of blood as possible.

Isolation of Tissues

Rabbit, cat, guinea pig, and human uterine muscle have been analyzed. In addition both guinea pig and rabbit taenia coli have been analyzed. In the case of the animal uteri, to permit analysis of relatively pure smooth muscle, endometrium was stripped off. The efficacy of this procedure was examined microscopically. Histological preparations of tissue isolated in this way indicated that cleavage usually occurred between the longitudinal and circular muscle layers. Occasionally a thin layer of circular muscle also was included, but in general the tissues analyzed contained only longitudinal muscle and a thin serosal covering.

Taenia coli was separated from intestinal circular muscle and mucosa without difficulty.

In Vitro

Some tissues (in which longitudinal muscle had been isolated as above) were analyzed after exposure during microelectrode studies for several hours in oxygenated Krebs–Ringer–bicarbonate. This medium was oxygenated with carbogen (95% O_2 – 5% CO_2) and warmed to 35° C.

Analytical Procedures

Methods for estimation of sodium, potassium, chloride, and water content in tissue and plasma samples have been previously described (9, 10, 11). Because of their small size, segments of guinea pig taenia coli were pooled for chloride analysis.

Symbols

The subscript t as in K_t indicates concentration in mM./kg. fresh tissue or ml./kg. in the case of H_2O. The subscripts c and e refer to concentration per liter of cellular and extracellular water.

Results

Control values for the electrolyte concentrations of these tissues and extracellular fluid are summarized in Table I. The sodium concentration in various types of smooth muscles is relatively high (70.7 to 74.7 meq./kg. fresh weight in taenia coli and 73.4 to 110.4 meq./kg. fresh weight in uteri). In contrast, tissue sodium concentrations of 15–35 meq./kg. for skeletal muscle and 42–50 meq./kg. for ventricle and 60–70 meq./kg. for auricular muscle have been observed in this laboratory in a variety of species (9, 11, 12). The smooth muscle potassium concentration was 89.1 to 90.6 meq./kg. in taenia coli and 55.8 to 79.0 meq./kg. fresh weight in uteri whereas values of 100–117 meq./kg. previously were found for skeletal muscle and 78–90 meq./kg. for ventricular muscle and 63 to 68 meq./kg. for auricular muscle (9, 11, 12).

The concentration of tissue water is high in most smooth muscle (782–790 in taenia coli and 766–843 ml./kg. fresh weight in uteri). Only the myometrium taken from cats during anestrus was in the lower range observed for skeletal muscle of a variety of species (750–775 ml./kg). Values for heart muscles were 818–819 ml./kg. for auricular muscles and 786–799 for ventricles.

The smooth muscle chloride concentration varied from 54 to 65 meq./kg. in taenia coli and 49 to 83 meq./kg. in uteri. In skeletal muscles, chloride values of 10 to 20 meq./kg. have been obtained and in cardiac muscles 31 to 43 meq./kg. for ventricular muscles and 55 to 64 for auricular muscles.

In summary, though the potassium concentrations of smooth muscle were generally less than those of striated muscles, the sodium concentrations were much higher and the sums of these cations (Na+K) were greater. This

TABLE I

WATER (ML./KG.) AND ELECTROLYTES (MEQ./KG.) IN VARIOUS TYPES OF SMOOTH MUSCLE

Animal	Condition	Tissue	No.		Na_t	Cl_t	K_t	H_2O_t	Na_e	Cl_e	K_e	H_2O_e
Cat	Anestrus	Uterus	6		110.4	70.6	59.3	765.5	155*	125*	4.2*	
				S.E.†	7.4	5.0	2.9	7.3				
	Estrogen	Uterus	11		94.0	76.4	63.7	806.1				
				S.E.	5.1	7.3	2.2	4.6				
	Estrogen and progesterone	Uterus	6		83.9	71.5	71.8	803.8				
				S.E.	4.7	2.2	2.7	4.9				
	Pregnant	Uterus	17		88.7	82.6	56.1	809.5				
				S.E.	2.7	2.3	2.1	4.7				
Rabbit	Estrogen	Uterus	12		79.1	57.0	73.9	843.4	145*	109*	4.5*	
				S.E.	2.0	1.3	1.5	4.0				
	Estrogen and progesterone	Uterus	9		73.4	49.1	79.0	829.8				
				S.E.	8.8	3.1	2.0	2.3				
	Pregnant	Uterus	1		75.0	69.3	71.4	818.0				
	(Male)	T. coli	5		74.7	54.0	90.6	789.6				
				S.E.	5.8	2.5	1.6	3.0				
Guinea pig	(Male)	T. coli	10		70.7	65.0‡	89.1	782.4	145*	109*	4.5*	
				S.E.	1.7		1.7	5.4				
	Estrogen	Uterus	1		78.4	60.9	73.8	819				
Human	Pregnant	Uterus	5		75.3	67.2	55.8	830.4	140*	112*	4.0*	
				S.E.	3.2	0.4	3.5	3.2				

*Averages for many samples analyzed in this laboratory after corrections for plasma solids and the Donnan equilibrium (19).
†Variability, of means in terms of standard error.
‡Value from pooled sample.

was the case even when these cation concentrations were calculated in terms of tissue water. In all respects auricular muscle most closely resembled smooth muscle in electrolyte and water composition. The variability in uterine muscle composition even in the same species reflects its responsiveness to varied hormonal environments. It was noteworthy that, in both cat and rabbit, progesterone treatment after estrogen decreased the sodium and chloride concentrations and elevated the potassium concentrations.

Distribution of Electrolytes

The higher sodium, chloride, and water concentrations of smooth muscle tissues indicated that the lower potassium concentrations might be the result of a large extracellular fluid volume (ECFV), rather than a lower potassium concentration in cells. Indeed a previous study (10) of whole uteri indicated that the chloride space varied from about 350 to 700 ml./kg. depending on species and prior treatment. The volume of distribution of chloride was considered the best approximation to the ECFV, since no evidence of chloride binding was obtained. However, this chloride space clearly overestimated ECFV in some smooth muscle (9, 13). Chloride probably is distributed in accordance with the electrochemical gradient in skeletal muscle (14) but little evidence is available regarding chloride distribution of smooth muscle. In Table II, the extracellular spaces of these tissues have been calculated on the basis of the following assumptions:

(1) That sodium is entirely extracellular and free to equilibrate with plasma water, i.e. calculation of sodium space.

(2) That chloride is entirely extracellular and free to equilibrate with plasma water, i.e. calculation of chloride space.

(3) That chloride and potassium are free to equilibrate across the cell membrane in accordance with a Donnan equilibrium, i.e. $K_c : K_e = Cl_e : Cl_e$.[2]

(4) That chloride passes freely across the cell membrane and is distributed in accordance with an electrochemical potential of 50 mv., i.e. the value found in many inactive smooth muscle cells (1–7) (Cl_e/Cl_c = antilog (0.050/0.061)).

TABLE II

VARIOUS ESTIMATES OF EXTRACELLULAR SPACES (ML./KG.) IN SMOOTH MUSCLE

Animal	Condition	Tissue	1 Na sp.	2 Cl sp.	3 ECFV K+Cl	4 ECFV E_{Cl}=50 mv.	5 ECFV E_{Cl}=35 mv.
Cat	Anestrus	Uterus	712	565	497	529	491
	Estrogen	Uterus	606	612	608	576	539
	Estrogen and progesterone	Uterus	541	572	568	530	487
	Pregnant	Uterus	573	656	628	634	606
Rabbit	Estrogen	Uterus	546	523	517	465	405
	Estrogen and progesterone	Uterus	506	450	442	382	311
	Pregnant	Uterus	517	636	632	603	568
	(Male)	T. coli	515	496	457	442	388
Guinea pig	(Male)	T. coli	488	596	593	563	527
	Estrogen	Uterus	541	559	554	512	463
Human	Pregnant	Uterus	538	600	585	558	529

[2] $ECFV = \dfrac{K_i Cl_i - (H_2O_i)^2 Cl_e K_e}{K_i Cl_e + Cl_i K_e - 2H_2O_i Cl_e K_e}$ if $\dfrac{K_c}{K_e} = \dfrac{Cl_e}{Cl_c}$.

(5) That chloride passes freely across the cell membrane and is distributed in accordance with an electrochemical potential of 35 mv., i.e. the interspike potential found in many active cat uterine cells (2, 4, 7) (Cl_e/Cl_c = antilog (0.035/0.061)).

All except the classical sodium and chloride spaces were dependent on the assumption of free chloride permeability with distribution according to various electrochemical gradients.

Several interesting facts emerge from such calculations. In all myometria of pregnant animals and in guinea pig taenia coli, the chloride space exceeded the sodium space. Even if the electrochemical gradient for chloride amounted only to 35 mv., with substantial quantities of tissue chloride assumed to be intracellular, the ECFV calculated on this assumption was usually as large as or larger than the sodium space. Myometrium from cats and guinea pigs treated with ovarian hormones also had somewhat larger volumes of distribution for chloride (at extracellular concentrations) than for sodium. The sodium space was larger than the chloride space only in myometrium from "anestrus" cats and from rabbits treated with ovarian hormones.

Cellular Ion Concentrations

In Table III, concentrations of intracellular sodium, potassium, and chloride have been calculated from the values for ECFV derived in Table II, employing the assumption that these ions were entirely in solution.

Any of the assumptions made for determining extracellular fluid volume leads to calculated concentrations of cellular potassium generally greater than those calculated for striated muscle (i.e. 140–170 meq./liter, see 14). Even with the method yielding the largest cellular volume (assumption 5 above) so that the cellular concentration is lowest, only three instances were encountered where the calculated cellular potassium concentration was less than 200 meq./liter. No value for potassium concentration less than 150 meq./liter of cellular water was encountered.

TABLE III

INTRACELLULAR CONCENTRATIONS OF SODIUM AND POTASSIUM (MEQ./L.) CALCULATED ON VARIOUS ASSUMPTIONS

			K_c					Na_c				Cl_c			
Animal	Condition	Tissue	1	2	3	4	5	2	3	4	5	1	3	4	5
Cat	Anestrus	Uterus	1161	284	213	241	208	114	120	120	161		2.5	19.2	33.6
	Estrogen	Uterus	306	315	308	266	230	−5	−1.5	25	39	3	1.7	19.2	33.6
	Estrogen and progesterone	Uterus	264	300	294	254	221	−21	−18	6	27	15	1.8	19.2	33.6
	Pregnant	Uterus	227	347	295	304	264	−149	−47	−55	−26	47	1.8	19.2	33.6
Rabbit	Estrogen	Uterus	240	223	220	190	164	10	12	31	47		2.2	16.7	29.3
	Estrogen and progesterone	Uterus	237	203	198	173	150	21	24	40	55		2.5	16.7	29.3
	Pregnant	Uterus	230	376	369	320	275	−95	−90	−58	−30	43	1.3	16.7	29.3
	(Male)	T. coli	322	301	266	248	221	10	25	29	46		1.8	16.7	29.3
Guinea pig	(Male)	T. coli	295	463	456	395	342	−85	−81	−50	−23	40	1.1	16.7	29.3
	Estrogen	Uterus	257	274	269	233	202	−11	−11	13	32	7	1.8	16.7	29.3
Human	Pregnant	Uterus	183	232	218	197	178	−38	−26	−10	4	24	2.1	17.2	36.1

Values for cellular sodium concentration varied a great deal irrespective of the assumption regarding chloride distribution which was used in their calculation. Negative values were calculated when the estimated ECFV exceeded the sodium space. These have been included in the table to provide an estimate of the magnitude of this discrepancy, and to permit calculation of the total cation per liter. The values for cellular sodium concentration should be compared to those found for striated muscle (10–25 meq./liter cell water).

Cellular chloride concentrations also have been calculated. When sodium space is assumed to equal the ECFV, substantial quantities of chloride were calculated to be present in cellular water. When potassium and chloride were assumed to be distributed in accordance with a Donnan equilibrium the estimated cellular concentration of chloride was very low. The calculated cellular chloride derived from this assumption thus approached the limits of analytical accuracy so that its existence would be difficult to ascertain. The remaining values indicate the rather large concentrations of cellular chloride which must be present if the potential measurements are accurate and if free permeability to chloride prevails.

Sum of Na and K Concentrations in Smooth Muscle

In Table IV, values for the sum of sodium and potassium in various portions of tissue have been assembled. The concentrations per liter of tissue water represent a minimum value, but even these levels were larger than similar values for plasma ultrafiltrate. Total monovalent cation concentrations ranged between 180 and 280 meq./liter, but values for anestrus and estrogen-treated cat myometrium and for taenia coli tended to be in the highest part of this range.

Effects of Exposure to Krebs–Ringer

In an attempt to study the relation of ion distribution and membrane potentials more accurately we performed electrolyte analyses on uterine muscle which had been studied with microelectrodes. These samples had been

TABLE IV

SUMS OF CONCENTRATIONS OF SODIUM AND POTASSIUM IN VARIOUS PORTIONS OF TISSUE WATER

Animal	Condition	Tissue	Na+K, meq./liter tissue H_2O	meq./liter cellular water					meq./liter plasma ultrafiltrate
				1	2	3	4	5	
Cat	Anestrus	Uterus	222	1161	398	333	361	333	159
	Estrogen	Uterus	196	306	310	306	291	269	
	Estrogen and progesterone	Uterus	186	264	279	276	260	248	
	Pregnant	Uterus	179	227	198	248	249	238	
Rabbit	Estrogen	Uterus	181	240	233	232	221	211	150
	Estrogen and progesterone	Uterus	184	237	224	222	213	205	
	Pregnant	Uterus	179	230	281	279	262	245	
	(Male)	T. coli	208	322	311	291	277	267	
Guinea pig	(Male)	T. coli	204	295	378	375	345	319	150
	Estrogen	Uterus	186	257	263	258	246	234	
Human	Pregnant	Uterus	157	232	194	192	187	182	144

stripped of extraneous tissue, suspended in Krebs–Ringer for 4–8 hours, and exposed to various stimulant drugs. Generally they remained in this medium for approximately 30 minutes after the final drug administration prior to removal for electrolyte determinations. The analytical data summarized in Table V indicate that considerable changes in the concentrations of tissue water and electrolytes had occurred. Extensive potassium loss and sodium gain occurred during the in vitro procedures. Sodium uptake approximately equaled potassium loss except in the case of human uterus. In this tissue excess sodium was gained so that the cation concentration increased. The tissue chloride and water concentrations also increased, but the chloride space usually expanded less than the sodium space as would be expected if part of the increased tissue sodium were due to exchange of this ion for cellular potassium. An exception was the human uterus in which the chloride space expanded more than the sodium space despite the marked uptake of sodium mentioned above. The possibility that these extensive changes were in part due to the procedures involved in stripping the longitudinal muscle must be considered. An attempt was made to estimate the effect of damage by comparing these data with those of intact samples of otherwise similarly treated animal uterus. The data summarized in Table VI indicate that similar but much less extensive changes occur in intact uteri in vitro. In general the sodium space expanded very little in cat tissues and less in all intact uteri than in comparable isolated longitudinal muscle. The increase in chloride space was similarly less in rabbit uteri. There were small decreases in potassium concentration. In the rabbit these were not equivalent to the increases in sodium concentration so that the sum of the concentrations of sodium and potassium per liter of tissue water was elevated. These results suggest that a substantial portion of the ion exchanges, particularly potassium depletion of longitudinal muscle, occurs as a consequence of the isolation procedure.

The thickness of human myometrium would prevent adequate diffusion into all portions of the tissue and therefore these have not been studied in the above fashion. Complete studies have not been made of taenia coli because the pieces used for microelectrode studies were too small for accurate analyses of all constituents.

TABLE V

ELECTROLYTE COMPOSITIONS OF PIECES OF LONGITUDINAL MUSCLE OF UTERUS BEFORE AND AFTER EXPOSURE TO KREBS SOLUTION

Animal	Condition	Na_t	Cl_t	K_t	H_2O_t	$\frac{Na+K}{l.H_2O_t}$	Na_{sp}	Cl_{sp}	$Na_{sp}-Cl_{sp}$	$Na_{sp}-Cl_{sp}$ control
Cat	Pregnant (3)	102.8	87.8	35.4	849	162.9	758	703	55	−83
Rabbit	Estrogen (1)	106.2	80.6	51.4	840	186.1	772	645	127	23
	Estrogen (1) and progesterone	121.9	100.7	40.0	859	188.4	886	806	80	56
	T. coli (3)	141.3	115.6	38.6	817	219.8	1045	934	111	19
Guinea pig	T. coli (2)	103.1	*	51.7	821	187.9	757	*	*	*
Human	Pregnant (5)	117.2	119.9	48.7	857	194.1	853	960	−107	−62

*In guinea pig taenia colon and in cat uteri the quantities of longitudinal muscle used often did not permit reliable analyses to be made.

TABLE VI

ELECTROLYTE COMPOSITIONS OF PIECES WITH WHOLE THICKNESS OF UTERINE WALL BEFORE AND AFTER EXPOSURE TO KREBS SOLUTION

Animal	Intact uterus, condition	Na_t	Cl_t	K_t	$\frac{Na+K}{l.\ H_2O_t}$	H_2O_t	Na_{sp}	Cl_{sp}	$Na_{sp}-Cl_{sp}$
Cat	Pregnant								
	Control*	81.6	77.7	42.2	153.9	805	527	621	−94
	Krebs–Ringer (1)	77.3	90.4	39.6	145.0	806	562	723	−161
Rabbit	Estrogen								
	Control*	79.8	51.1	67.7	175.5	840	551	468	83
	Krebs–Ringer (6)	97.4	74.4	68.0	195.0	848	708	595	113
	Estrogen*+progesterone								
	Control*	75.0	47.7	68.3	175.7	816	517	505	12
	Krebs–Ringer (5)	92.7	72.7	62.2	184.4	839	674	582	92

*See reference 10.

Discussion

The High Concentration of Cation in Smooth Muscle

There are two possibilities to consider in attempting to provide an explanation of the high values for the sum of sodium and potassium concentrations in smooth muscles. Either there was a true hyperosmolarity of cellular or interstitial fluid compared with plasma or there was not. A true hyperosmolarity could result (1) from active transport of water, (2) from active transport of ions and impermeability to water, or (3) from the existence of non-diffusible anions or other ions which set up a Donnan equilibrium. The nerve sheath has been postulated to set up a Donnan equilibrium which accounts in part for the finding of a high sum of sodium plus potassium concentration in cat nerves (17). It seems unlikely that hyperosmolarity with respect to interstitial fluid due to a Donnan equilibrium ever exists in cellular water of animal cells with non-rigid walls. In addition, no evidence for hypertonicity of interstitial or cellular fluid has been found in smooth muscle. For example, previous studies have indicated that 167 m*M* choline chloride and 333 m*M* sucrose caused a net loss of water from whole uterus (10), but this may have been secondary to diffusion of sodium or potassium along with anions from tissue fluids inaccessible to sucrose or choline. On the other hand, as indicated in Table VI, when whole uteri were exposed to Krebs–Ringer, which contains approximately 300 milliosmoles of ionized solutes and 49 milliosmoles of glucose, they increased their water concentration somewhat, and gained weight (less than 10%). However, sodium and chloride spaces also expanded, particularly the former. This sodium gain was not accompanied by marked potassium leakage and probably represented chiefly intake into interstitial spaces by swelling and by appearance of new relatively non-diffusible anionic charges. The sodium and chloride spaces would expand equally during water gain of interstitial fluid by swelling. To explain the preferential expansion of the sodium space appearance of new anionic charges would have to be postulated. The increase in the sodium space of tissues in Krebs solution was in fact generally sufficient to account for the water gain. It appears, then, that the osmotic

concentration of uterine fluids was not much less than 300 milliosmoles/liter or more than 333 milliosmoles/liter. These results provide no evidence of hyperosmolarity of tissue fluids in the uterus. Though there is still some disagreement as to the evidence, the best measurements (see 14) seem to indicate that the freezing point depressions of cellular and interstitial fluids are identical with those of a plasma ultrafiltrate provided autolytic changes have been prevented. This suggests that active water transport or impermeability or in fact, hyperosmolarity due to any cause probably does not exist in most mammalian tissues. Hence it seems valid to attempt to explain the concentration of univalent column measured here in terms of isosmotic conditions in tissue fluids.

Two such possibilities can be considered. First, a portion of cellular or extracellular anions may be polyvalent so that achievement of isosmolarity and of electrical neutrality would require a relative excess of univalent cations. Second, the observed cation concentrations may be distorted by the inclusion of bound osmotically inactive sodium or potassium. For the second of these possibilities some evidence is already in existence.

In both intact uteri and in isolated longitudinal uterine muscle (see 7, 10, 16) a substantial fraction of tissue sodium (5–10 meq./kg. fresh weight for rabbit and 10–20 meq./kg. fresh weight for cat) remains in the sample, after prolonged (4 hours) immersion in sodium-free solutions. Sodium binding would readily account for these results. Furthermore, the amount of potassium lost over a similar interval from uterine tissue into potassium-free solutions (whether sodium was present or not) was extremely dependent upon temperature. Reduction of temperature from 37° to 25° resulted in approximately a twofold decrease in the rate of potassium loss from tissues bathed in potassium-free medium (10, 15). Decreased potassium outflow at reduced temperature would be difficult to explain if potassium were entirely in solution since a substantial increase in the resting potential or decrease in permeability to outflow of potassium would be a necessary postulate. Decreased potassium outflow could be readily explained if the rate of loss of potassium were dependent on a chemical process which unbinds potassium or utilizes polyvalent anion. Conclusive evidence of cation binding must await studies of exchange of radioisotopes.

In clarifying this problem for future investigation, it seemed worthwhile to try to assign quantitative values to the demands of these two hypotheses. In Table VII, (A) and (B), such values have been calculated, assuming in (A) that the sums of univalent concentrations were attributable to polyvalent cellular anions, and in (B) that they were due solely to cation binding. Certain simplifications have been made. (1) Polyvalent anions have been assumed to be diffusible to avoid the complications of a Donnan equilibrium and because no evidence for true hyperosmolarity exists. (2) Polyvalent ions such as Ca^+ and PO_4^- in the extracellular fluid were ignored since these would not alter the calculated values to an appreciable extent. (3) In addition, all positively charged particles other than Na^+ and K^+ which might exist in the cellular

water (14) have been ignored since their quantities, though likely small, are unknown in smooth muscle and since their inclusion only increases the extent of the excess cellular positive charge which requires explanation. (4) Activities of all ions in various media have been assumed to be equivalent to their concentrations. Equations similar or identical with them have been derived on similar assumptions by Conway (14) and on somewhat different assumptions by Hodgkin (18).

TABLE VII

ANION VALENCIES AND CATION BINDING REQUIRED TO ACCOUNT FOR CATION CONCENTRATIONS OF SMOOTH MUSCLE*

			A					B $(Na+K)_{bound}$ per:		
			Na_c+K_c	Cl_c	$An_c Z_{an}$	An_c	Z_{an}	Liter H_2O_t	Liter cell water	Kg. DS
Cat	Anestrus	Uterus	334	17	317	0		63	175	208
	Estrogen	Uterus	271		254	45	5.6	37	112	164
	Estrogen +progesterone	Uterus	251		234	65	3.6	27	69	111
	Pregnant	Uterus	240		223	76	2.9	20	80	85
Rabbit	Estrogen	Uterus	210	14	196	109	1.8	31	60	166
	Estrogen +progesterone	Uterus	204		186	115	1.6	34	54	166
	Pregnant	Uterus	245		231	74	3.1	29	95	130
	T. coli	Uterus	262		248	57	4.4	58	114	218
Guinea pig	T. coli		317	14	303	2	151.5	54	166	194
	Estrogen	Uterus	232		218	87	2.5	36	83	163
Human	Pregnant	Uterus	180	15	165	138	1.3	13	36	63

The last column in Table VII (A) indicates the average charge per mole of intracellular anions (Z_{an}) required to fulfil the hypothesis of hypertonicity due to polyvalent cellular anions. The values range, with two exceptions[3] between 1.3 and 5.6. These values for Z_{an} would be decreased to the extent that binding of Na^+, K^+, and Cl^- occurs. Maximum values as low as these suggest that ions of moderate size and charge such as phosphate esters and organic acids contribute substantially to the process and that highly polyvalent anions play a minor role. However, it is of interest that the maximum average valencies of anions reported to exist in skeletal muscle (14) were all less than 1.87 so that different compounds might be required to explain the excess cation of smooth muscle.

These are maximal values only in one sense. By reference to equation 4 (see Appendix) the effects of other positive charges in the cell become apparent since the quantities Cl_c, Na_c, and K_c are calculated independently; to add another quantity to the values already known would decrease the quantity of An_c, the moles of polyvalent non-diffusible anion. This would increase the

[3]In the two exceptions, anestrus cat myometrium and guinea pig taenia colon, higher values were calculated owing to the fact that the sum of the values for $Na_c+K_c+Cl_c$ was near to or greater than the value for total extracellular osmolarity. This resulted primarily from exceptionally high values of Na_c+K_c, which in turn was a product of the combination of high values for $Na+K/H_2O_t$ and ECFV. In these instances ion binding of cation and/or chloride must have occurred.

valency calculated for these anions, up to the point where $Na_e+K_e+Cl_e+$ $Anion_e$ became equal to or greater than 333. Thereafter, the only acceptable explanation would be ion binding. Similarly, it is apparent that if there are other cellular cations than $Na^+ + K^+$ in significant quantities, then equation 1 is incorrect in that Na_e+K_e would be less than Na_e+K_e in those quantities. The listed values for Z_{an} are in this sense a minimal figure. However, this consideration only tends to reinforce the possibility that Z_{an} may be larger in these tissues. It seems unlikely, however, that substantial numbers of additional positive charges exist in addition to the excess of $Na^+ + K^+$.

In Table VII (B) the amounts of monovalent cation which would be bound if hypertonicity were due exclusively to cation binding have been calculated with respect to tissue water, cellular water, and dry solids (DS). The latter is the most rational form of expression in this case, and therefore it was of interest that such calculations led to a rather consistent ordering of the data. Thus smooth muscles which were not exposed to ovarian hormones (anestrus cat) or did not respond to them (taenia coli) invariably had values for total bound tissue monovalent cations in the range of 194–218 meq./kg. DS. Those uteri which were experimentally treated with estrogen, or estrogen and progesterone with one exception (111 meq./kg.), possessed total bound tissue cation concentrations in the range of 154 to 166 meq./kg. DS. Uterine smooth muscle from pregnant animals whose uteri had been exposed to ovarian hormones for longer periods had even lower values for bound sodium and potassium ion (63–130 meq./kg. DS).

Binding of chloride must also be considered as a possibility although available evidence (10) suggests that chloride is freely diffusible from these tissues. Uteri from spayed rats have recently been reported to contain a high proportion (10%) of collagen. Estrogen and estrogen plus progesterone treatment decreased this proportion by causing growth of the tissue without collagen formation (19). A higher proportion of collagen also occurs in pregnant rats (20). Collagen has been reported to bind chloride, but not sodium (21), though there is disagreement on this point (22, 23). The binding of chloride by collagen might influence the results by causing a falsely high ECFV when this is calculated using some assumption as to chloride distribution. In the case of pregnant uteri, the excess of chloride space over sodium space might result wholly or in part from chloride binding. However, this will not remove the problem of the high value of sum of tissue sodium and potassium concentration except as it results in a calculated increase in CFV. This, of course, would not affect the calculation of sum of sodium and potassium per liter of tissue water, which as pointed out above, leads to values higher than those of plasma. The residual sodium found in whole uteri would account for only about 50–100 meq./kg. DS (10), so that potassium binding probably should be considered as a possible contribution to these relations.

Chloride and Potassium Distribution

The evidence presently available still permits the hypothesis that smooth muscle chloride is freely diffusible across the cell membrane and probably

distributed at or near its equilibrium potential. However, this same conclusion cannot be drawn with respect to potassium. The lowest calculated potassium concentration ratio across the cell membrane (150/4.5 = 33.0) leads to the prediction of an equilibrium potential of approximately 93 mv. at body temperature. No potential of this magnitude has so far been reported in smooth muscle (1–7) and the observed values average 50 mv. It seems justifiable to assume that no net flux of ions was occurring in the uterus in vivo. Similar values for membrane potential have been measured in vivo (4) and in vitro (7). Therefore, the relationships developed by Conway (14) for the balanced state should apply and permit comparison between the equilibrium potential and the measured potential. Either potassium is actively transported across these membranes or the ratio has been distorted by the inclusion of bound potassium in the apparent intracellular concentrations of potassium.

In myometrium treated with ovarian hormones, the maximal amount of bound potassium that conceivably could be present (assuming that all hypertonicity were due to potassium binding) averages about 69 meq./kg. of cell water. When this value is subtracted from the average figure for each of these tissues (211 meq./liter—see column 5 of Table III) the net ratio falls to 142/4.5 = 32. If the minimal value from this column is used, the ratio becomes 81/4.5 = 18. The equilibrium potentials for these potassium concentration ratios would be 92 and 77 mv. respectively, still greater than the potentials recorded. Therefore, it seems extremely likely that active transport of potassium occurs in these tissues. It might be objected that tissue potassium loss occurs in vitro and vitiates the comparisons based on the assumption of no net ion fluxes. However, the data in Tables V and VI indicate that most of the potassium loss in vitro was the result of damage in isolation of tissues since it occurred in those subjected to separation of the longitudinal muscle layer. Low values for membrane potential are usually attributed to cell damage and such measurements generally are not accepted in calculating mean values for transmembrane potential so that a fair comparison would be based upon the potassium distribution in undamaged cells. However, even when damaged cells were included, and K_c calculated from data in Table VI the values exceed 125 meq./liter in these tissues after several hours in the Krebs solution. Here as well, the evidence suggests that the potassium ion is not distributed according to its equilibrium potential and that the differential is not maintained by any mechanism requiring leakage of potassium.

Sodium Distribution

Sodium distribution, as in other tissues, requires the postulation of either membrane impermeability or active sodium extrusion. Data to be reported subsequently indicate that whole uteri, on exposure to potassium-free Krebs solution do take up sodium and lose potassium and, on return to simple solutions containing some potassium salts, this process is reversed. Therefore, active sodium transport probably is the mechanism whereby sodium is extruded from smooth muscle. The extensive uptake of sodium into damaged cells makes this difficult to demonstrate conclusively in similar experiments in tissue dissected to provide "pure" smooth muscle.

Other Data

The above discussion clearly demonstrates the importance of data on the extent of ion binding for accurate calculation of the actual electrochemical gradients of smooth muscle. Data on the ion fluxes across the smooth muscle membrane, in combination with knowledge of the membrane potential, would be of great value for determining the mechanisms of ion distribution in these tissues.

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Appendix

Results in part A of Table VII calculated by use of the following equations.

$$[1] \qquad (Na_c+K_c)\frac{CFV}{H_2O_t}+(Na_e+K_e)\frac{ECFV}{H_2O_t}=\frac{Na_t+K_t}{H_2O_t}$$

This equation states that all the tissue sodium and potassium were dissolved in either cellular or extracellular fluid. ECFV was calculated assuming that $E_{Cl}=35$ mv. Other values were substituted and the equations solved for Na_c+K_c ion concentrations in meq. per liter or kilogram H_2O_t; ECFV and CFV in liters per kilogram.

$$[2] \qquad E_{Cl}=0.061 \log \frac{Cl_e}{Cl_c}$$

This equation assumes free permeability to chloride and no binding of this ion. It was also assumed that $E_{Cl}=35$, and the equation was solved for Cl_c.

[3] $$Cl_c + An_c \cdot Z_{an} = Na_c + K_c$$

This equation assumes that the principle of electroneutrality holds.

Z_{an} = no. charges per mole of cellular anions. It was solved for $An_c \cdot Z_{an}$.

[4] $$Cl_c + An_c + Na_c + K_c = 333$$

This equation assumes that the membranes of these cells are freely permeable to water, so that the sums of millimoles or milliosmoles per liter of fluid are the same in both cellular and extracellular fluid; extracellular fluid assumed to contain 333 osmoles/liter (14). It was solved for An_c.

[5] $$\frac{An_c Z_{an}}{An_c} = Z_{an}$$

Results in part B of Table VII calculated by use of the following equations:

[6] $$K_c + Na_c = K_e + Na_e$$

This equation assumes that there are no polyvalent cellular anions and that the sums of univalent cations are identical on both sides of the membrane.

[7] $$\frac{Na_t + K_t}{H_2O_t} - \frac{(K+Na)_{bound}}{H_2O_t} = (K_c + Na_c)\frac{(1-ECFV)}{H_2O_t} + (K_e + Na_e)\frac{(ECFV)}{H_2O_t}$$

This equation states the identity between the sums of the concentrations of free sodium and potassium in tissue and the fractions of each of these ions which are in the cellular and extracellular fluids.

[8] $$\frac{K_t + Na_t}{H_2O_t} - (K_e + Na_e) = \frac{(K+Na)_{bound}}{H_2O_t}$$

This equation was obtained by substituting [6] into [7], rearranging, and solving for $\frac{(K+Na)_{bound}}{H_2O_t}$.

[9] $$\frac{(K+Na)_{bound}/H_2O_t}{1-\frac{ECFV}{H_2O_t}} = \frac{(K+Na)_{bound}}{\text{liter cell } H_2O}$$

This equation permits calculation of the bound monovalent cation in terms of cellular water instead of tissue water.

[10] $$\frac{(K+Na)_{bound}/H_2O_t}{\frac{1-H_2O_t}{H_2O_t}} = \frac{(K+Na)_{bound}}{\text{kg. dry solids}}$$

This equation permits calculation of the bound monovalent cation in terms of dry tissue solids.

STUDIES ON HUMAN ADRENAL STEROIDS

2. ISOLATION OF 20α-DIHYDROCORTICOSTERONE FROM THE PLASMA OF NORMAL MALES TREATED WITH CORTICOSTERONE[1]

C. M. SOUTHCOTT, V. A. SPROULE, HAMISH MCINTOSH, AND MARVIN DARRACH

Abstract

Five fasting normal male humans were infused intravenously with 200 mg. of corticosterone. Blood samples were then withdrawn at various time intervals and the plasma was analyzed for individual Δ^4-3-keto C_{21} adrenal steroids. In each case 20α-dihydrocorticosterone appeared as a metabolite. The isolated corticosterone and 20α-dihydrocorticosterone were characterized chemically and quantitative plasma levels of each are reported.

In a previous report it was shown that the administration of corticosterone to the mouse resulted in increased plasma levels of 20α-dihydrocorticosterone (Δ^4-pregnene-11β,20α,21-triol-3-one) (6). The reduction, in vivo, of the C-20 keto group of corticosterone to an α-hydroxyl group has now been observed in the human. The present communication describes the isolation of 20α-dihydrocorticosterone from plasma following intravenous infusions of corticosterone into normal male humans.

Experimental

Administration of Corticosterone, Collection of Blood, Extraction from Plasma, and Chromatography of the Neutral Unconjugated C_{21} Adrenal Steroids

In each of five experiments a control specimen of about 20 ml. of peripheral blood was withdrawn at 8.00 a.m. from a fasting normal male donor. Then 200 mg. of corticosterone, which had been dissolved in 10 ml. ethanol and diluted with 500 ml. of 5% dextrose,[2] was infused intravenously for 30 minutes. Blood samples were taken at specified time intervals following the infusion, mixed with known amounts of dextrose sodium citrate solution,[3] and centrifuged. The plasma fractions were frozen and stored at −2° C. As described previously (5) the neutral unconjugated C_{21} adrenal steroids were isolated from the plasma by dialysis, chloroform extraction, and paper chromatography.

Characterization of 20α-Dihydrocorticosterone and Corticosterone

Figure 1 shows chromatograms prepared from the plasma fraction of blood withdrawn from one patient 30 minutes after an intravenous infusion of corticosterone. As described below, the zones at F 0.6 and B 1.0 contained 20α-dihydrocorticosterone and corticosterone respectively. The plasma control taken before the infusion yielded neither F 0.6 nor B 1.0 zones.

Ultraviolet (U.V.) absorbing zones at F 0.6 and B 1.0 also appeared on the chromatograms prepared from the 30-minute post infusion specimens withdrawn from the other four patients. In each case the F 0.6 and B 1.0 zones

[1]Manuscript received April 19, 1958.
Contribution from the Department of Biochemistry, University of British Columbia and Clinical Investigation Unit, Shaughnessy Hospital, Vancouver, Canada.
[2]Travenol—5% dextrose w/v in water, Baxter Laboratories of Canada Ltd., Acton, Ontario.
[3]Dex-trate—Formula No. 1, Baxter Laboratories of Canada Ltd., Acton, Ontario.

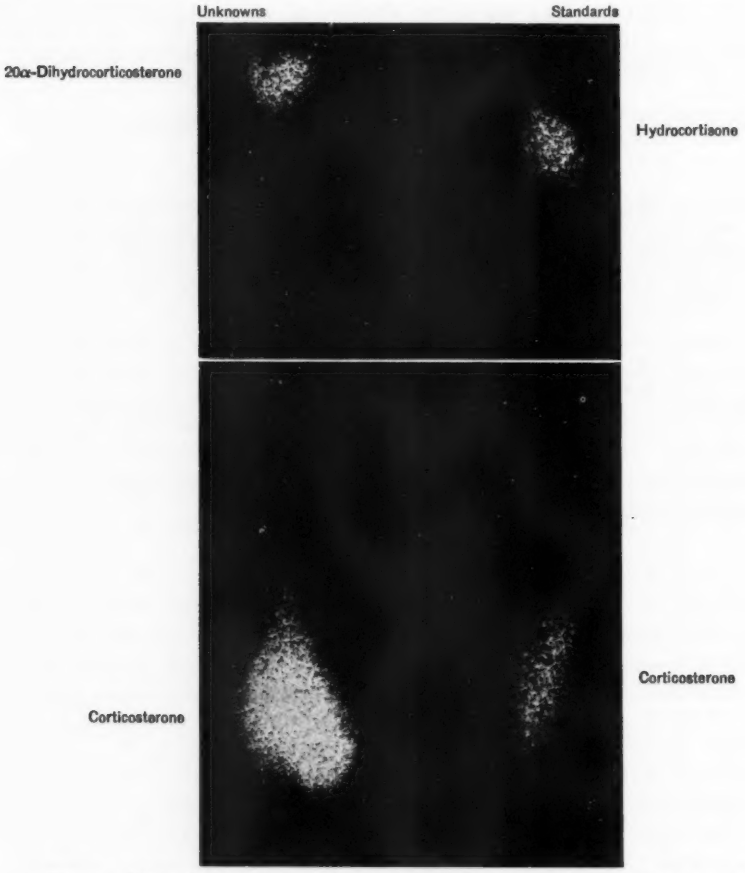

FIG. 1. Ultraviolet contact photographs of chromatograms prepared from human plasma 30 minutes after an intravenous infusion of corticosterone.

were eluted with methanol and analyzed quantitatively for Δ^4-3-keto steroids by the ultraviolet method (5). These data are presented in Table I. The eluates of the F 0.6 and B 1.0 zones were combined separately, taken to dryness at 40° C. under nitrogen, and the contents characterized chemically.

F 0.6 zones (*20α-dihydrocorticosterone*).—The pooled sample yielded a single U.V. absorbing zone after paper chromatography for 5 days in the toluene – propylene glycol system of Zaffaroni (7). The substance at the F 0.6 zone exhibited a U.V. absorption maximum at 242 mμ in ethanol, a negative blue tetrazolium (B.T.) reaction, and a sulphuric acid absorption spectrum with maxima at 285 and 340 mμ. After 5 days on a toluene – propylene glycol chromatogram it did not separate from authentic 20α-dihydrocorticosterone. The product of mild acetylation descended in the benzene–formamide system at the same rate as 20α-dihydrocorticosterone

diacetate. These data indicated the configuration of the compound at the F 0.6 position to be that of 20α-dihydrocorticosterone (Δ^4-pregnene, 11β, 20α, 21-triol-3-one) (6).

TABLE I

CORTICOSTERONE AND 20α-DIHYDROCORTICOSTERONE LEVELS IN HUMAN PLASMA 0.5 HOUR AFTER INTRAVENOUS INFUSIONS OF 200 MG. CORTICOSTERONE (GAMMA/100 ML. PLASMA—AS FOUND)

Patient	Corticosterone	20α-Dihydrocorticosterone
1	60.5	9.0
2	97.6	15.1
3	75.6	10.2
4	62.0	14.2
5	53.0	7.4

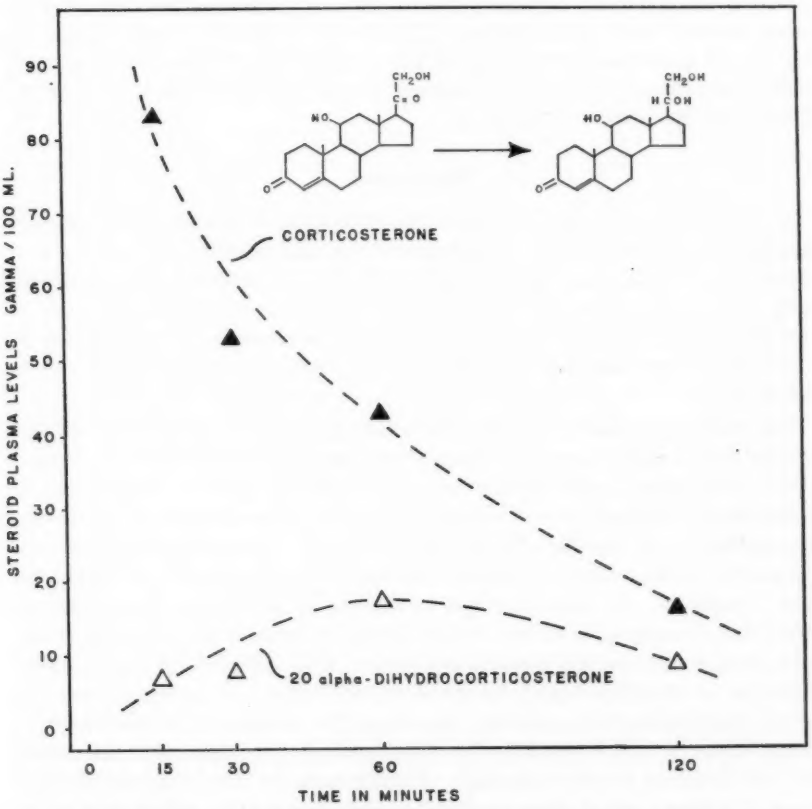

FIG. 2. Corticosterone and 20α-dihydrocorticosterone levels in human plasma following an intravenous infusion of 200 mg. of corticosterone.

B 1.0 zone (*corticosterone*).—The pooled sample was chromatographed in benzene–formamide and the substance at the B 1.0 zone was immediately eluted with ethanol. It gave a U.V. absorption maximum at 242 mμ and a positive B.T. reaction. The ratio of values, given by the quantitative U.V. and B.T. methods and determined as corticosterone against a corticosterone standard, was 1.00:1.01. In sulphuric acid the absorption curve showed maxima at 288, 328, 373, and 454 mμ. The compound did not separate from authentic corticosterone in benzene–formamide, and after mild acetylation its derivative descended at the same rate in benzene–formamide as authentic corticosterone acetate. Thus the compound at the B 1.0 position proved to be recovered corticosterone (Δ^4-pregnene,11β,21-diol-3,20-dione).

Plasma Levels of 20α-Dihydrocorticosterone and Corticosterone at Various Time Intervals Following an Intravenous Infusion of Corticosterone

In one case the blood samples withdrawn at 15, 30, 60, and 120 minutes following the end of the corticosterone infusion were analyzed for 20α-dihydrocorticosterone and corticosterone. The F 0.6 (20α-dihydrocorticosterone) and B 1.0 (corticosterone) areas of the chromatograms derived from each plasma sample were eluted and analyzed quantitatively by the U.V. method. These data are presented in Fig. 2.

Discussion

In the human (3,4), as in all other mammals thus far examined, corticosterone appears to be a normal constituent of the adrenal secretion. Thus it is important to understand the manner in which this hormone is altered chemically in the extra-adrenal tissues. Such information reveals the presence of enzyme systems capable of inactivating the molecule and directs attention to biochemical reactions which may be related to basic mechanisms of action of the hormone.

The biological reduction of the C-20 ketone group of several adrenal steroids in a variety of experimental conditions has been the subject of recent reviews (1,2). The experiments reported here demonstrated that in the human, as in the mouse, the product of C-20 keto reduction of corticosterone circulating in peripheral blood was 20α-dihydrocorticosterone. These data do not exclude the possibility that 20β-dihydrocorticosterone was also formed in certain tissues. However, the amount of this compound in the plasma, if any, was so small that it escaped detection by the methods employed. Although it appears that in the normal human and mouse, the C-20 keto group of corticosterone is reduced primarily to an α-hydroxyl group, the evidence does not permit conclusions to be reached concerning the orientation of the hydroxyl group following C-20 reduction of corticosterone in other species or the nature of C-20 reduction of other steroids. Furthermore, in vitro experiments may create conditions which show qualitative and quantitative differences in the C-20 reduction products from those observed in vivo.

Acknowledgments

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DISUSE ATROPHY OF SKELETAL MUSCLE IN THE RAT, AGGRAVATED BY CORTISOL AND VARIOUS STRESS CONDITIONS[1]

EÖRS BAJUSZ

Abstract

Slight atrophy of the triceps surae muscle of the rat was produced by immobilization of one hind limb in a plaster of Paris cast. The development of disuse atrophy was significantly aggravated when the immobilized animal was exposed to stressors and/or treated with cortisol. These results indicate that immobilization of one limb, which did not produce any visible local damage through compression, can act as a "conditioning" factor which selectively sensitizes the muscular tissue to the catabolic effect of endogenous and exogenous corticoids. It is probable that the conditioning is due to a circulatory disturbance caused by the immobilization, but this assumption has not been definitely proved. These experiments also indicate that the type of immobilization used here can act as a "neurogenic" stressor agent, and by thus producing general catabolism predisposes the muscular tissue for the development of a greater degree of atrophy.

Introduction

The study of muscle atrophy has absorbed the interest of a large number of experimental and clinical workers (5, 7, 11, 14, 15, 23), but the problem is still far from being solved. Three classical types have been established as a result of studies concerning the production and mechanism of atrophy in muscle: (1) muscle atrophies of nervous origin (e.g. denervation, upper or lower motor neuron lesions, deafferentation, lesions of the central nervous system), (2) disuse atrophies in muscles, due to inactivity (e.g. skeletal fixation, immobilization with plaster cast), and (3) muscle atrophies due to tenotomy.

The atrophy and degeneration of denervated skeletal muscle have generally been held to constitute the outstanding demonstration of trophic control of a non-nervous tissue by the nervous system. The atrophy which results when an innervated muscle is held at a fixed length by immobilization is a different process from that which occurs in denervated muscle. In atrophy of innervated muscle, there is no fibrillation and no fatty replacement, and the motor endplates are the last, rather than the first, structures to be affected. The rate of atrophy from disuse is similar to, but not so profound as, that caused by denervation. The atrophy due to tenotomy is, in part, an atrophy of inactivity, but it must be taken into consideration that, in the tenotomized muscle, myostatic contracture develops.

In this laboratory, it was previously observed that if a vasoligature procedure is followed by the daily administration of cortisol, both extremities of the rat undergo some involution as a result of the hormone's catabolic effect; however, the vasoligated side is specifically sensitized to this action (19). Subsequent work clearly showed that the catabolism which normally occurs during stress or as a result of cortisol administration can be selectively increased in an

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Contribution from the Institut de Médecine et de Chirurgie expérimentales, Université de Montréal, Montreal, Canada.

individual muscle sensitized by experimentally produced vascular deficiency (1, 2). These experiments revealed the hormonal production of local muscle atrophy of an innervated and well-functioning area in which an interference with the circulation, too slight to produce any manifest disturbance in itself, "selectively conditioned" the muscular tissue to the catabolic effect of cortisol treatment or of blood-borne corticoids. Other observations indicated that the atrophy of a skeletal muscle which follows severance of its motor nerves, can be further enhanced by certain hormones which produce intense catabolism (e.g. cortisol, estradiol, thyroxin), and delayed by an anabolic hormone, such as STH (18).

From these findings, it seemed necessary to investigate further the role of hormones in the development of muscle atrophy. In the following we report an experimental series in which we studied the effect of diverse conditions of stress and of cortisol administration upon the development of disuse atrophy in the rat.

Experimental Materials and Techniques

General Experimental Design

One hundred young female Sprague–Dawley rats, with an average initial body weight of 86 g. (range: 82–92 g.), were subdivided into 10 equal groups, and treated as indicated in Table I.

The rats of *Group I* were killed the day the experiment started, to serve as absolute controls showing the normal initial values. The rats of *Group II* received no treatment, but one hind limb of each animal in *Group III* was immobilized in a plaster cast, as described below. At the end of the experi-

TABLE I

DISUSE ATROPHY OF SKELETAL MUSCLE IN THE RAT AGGRAVATED BY CORTISOL AND VARIOUS STRESS CONDITIONS

Group	Treatment			Final body weight, g.	Triceps surae, mg.		
	Plaster cast	"Stressor"	COL-Ac		Intact	Disused	%*
I		Untreated (initial)		86±0.9	415± 4.2	(415±3.9)†	100
II		Untreated (final)		142±2.9	939± 4.9	(939±4.7)†	100
III	Plaster cast	—	—	114±2.2	670± 8.7	558± 5.7	83.2
IV	Plaster cast	—	COL-Ac	73±2.3	393±12.4	237± 9.7	60.5
V	Plaster cast	Partial fasting	—	70±1.6	373± 9.9	268±11.9	71.8
VI	Plaster cast	Partial fasting	COL-Ac	68±2.1	320± 7.0	175±10.9	54.6
VII	Plaster cast	Exposure to cold	—	92±2.4	514± 8.5	383± 9.2	74.5
VIII	Plaster cast	Exposure to cold	COL-Ac	85±5.1	397±10.7	214± 9.7	53.9
IX	Plaster cast	Formaldehyde	COL-Ac	77±2.2	404±13.9	229±13.8	56.6
X	Plaster cast	Restraint	COL-Ac	73±1.8	344±12.2	269±11.0	78.1

*The weight of the intact muscle is taken as 100%.

†In the untreated controls (Groups I and II) the left limb was not immobilized; the values are given here only for comparison with those of the other groups in which the corresponding hind limb was immobilized with a plaster cast, as described in the text.

ment, the animals of these groups were killed and they served as untreated controls showing the final normal values (Group II), and the change in the somatic growth and the degree of the atrophy of the triceps surae muscle caused by immobilization with the cast alone (Group III). The left hind limbs of all rats in the *Groups IV–X*, also, were immobilized in a plaster of Paris cast.

Throughout the period of observation (21 days) the animals were fed exclusively on ground laboratory diet, "Purina Fox Chow", and tap water ad libitum. The food was ground, otherwise the rats would have found difficulty in eating it as the teeth were clipped every 3 days to prevent the immobilized animals from gnawing the plaster cast around the leg. The controls whose teeth were also clipped received the same ground food.

The Production of Disuse Muscular Atrophy

For the production of disuse atrophy, the left hind limb of the animal was immobilized in a plaster of Paris cast. The bandage was wound tightly around the layer of absorbent cotton and, to prevent the animal from freeing his limb, the cast was stitched to the skin of the hip and the ankle. The limb was immobilized in the extended position, but the hip joint and the toes were allowed to move freely so that the animal was able to walk with a stiff gait. No damage due to compression of the cast (e.g., edema, ischemia) nor necrosis of the skin due to the stitches was observed. The procedure was carried out, under light ether anesthesia, on the first day of the experiment. All additional treatments were begun on the following day.

The Production of Diverse Conditions of Stress

In order to produce diverse conditions of stress, we used four different noxious agents (or "stressors"), i.e., partial fasting, exposure to cold, restraint, and treatment with formaldehyde.

(*a*) *Partial fasting.*—This was accomplished (Groups V, VI) by reducing the food intake. The rats of these groups were kept in separate cages and received 6–8 g. of the ground "Purina Fox Chow" daily. Previous experiments showed that reducing the food intake in such a manner makes the normal 80–100 g. rat lose body weight at the average rate of 1 g. per day (20).

(*b*) *Exposure to cold.*—The animals of Groups VII and VIII were exposed to cold, by being kept at +3° C. every second day for a period of 14 hours, until the termination of the experiment.

(*c*) *Treatment with formaldehyde* (*Group IX*).—The rats of this group received 0.5 ml. of 2% formaldehyde, subcutaneously, daily.

(*d*) *Restraint.*—Restraint, e.g. neuromuscular strain, was induced by strapping the rats of Group X on a board with adhesive tape, for a period of 7 hours every second day, until termination of the experiment.

Cortisol acetate (COL-Ac) was administered in the form of microcrystals of "hydrocortisone acetate" (Schering, Montreal), at the dose of 1 mg./day in 0.2 ml. of water, subcutaneously, until the termination of the experiment (Groups IV, VI, VIII, IX, and X).

The Evaluation of the Experiment

On the 21st day after the initiation of treatment, all rats were killed with chloroform, and the triceps surae muscles were dissected. After fixation in Susa solution, the muscles were weighed on an analytical balance. The means of these weights, as well as of the final body weights, are indicated in Table I, together with the standard errors. The differences between the contralateral muscles are especially indicated in a separate column, in which the weight of the muscle of the disused side is expressed as a percentage of the weight of the corresponding muscle on the intact side. Following the weighings, the muscles were embedded in paraffin and stained with hematoxylin–phloxine for subsequent histologic study.

Results

The results of the experiments are summarized in Table I. It will be seen from the figures given in this table that immobilization alone produced diminution in the rate of *somatic growth* (cf. Group III). This diminution was more marked in animals submitted to immobilization together with stressors and/or treatment with COL-Ac (Groups IV–X).

Comparison of the muscle weights of the *intact side* with the final body weights shows that, in animals immobilized in a plaster cast, with or without exposure to various stressors and/or COL-Ac: (1) the decrease in the intact triceps surae muscle weight parallels that of the body weight, (2) the diminishment of the triceps surae muscle growth parallels that of the somatic growth.

On the other hand, the weights of the triceps surae muscles of the *disused side*, in the rats treated with COL-Ac and/or various stressors (Groups IV–X), are decreased to below the initial values (compare with Group I).

Immobilization alone produces only slight muscle atrophy (Group III; 83.2%), although in these animals the somatic growth and growth of the triceps surae muscle were markedly diminished on both the intact and disused sides. When immobilization was followed by COL-Ac administration (Group IV), or by diverse conditions of stress alone (Groups V and VII) or with COL-Ac (Groups VI, VIII, IX, X), increased catabolism in the triceps surae muscle of the disused side became evident (see Table I). The figures given in Table I also indicate that when exposure to various stressors is combined with COL-Ac administration, the resulting disuse atrophy of the triceps surae muscle is more marked (compare Groups VI, VIII with Groups V, VII). There is no marked variation in the degrees of muscle atrophy caused by various stressors (71.8%, 74.5%); the addition of COL-Ac (Groups VI, VIII, IX) produced a greater degree of atrophy which was still independent of the type of stressor used (54.6%, 53.9%, 56.6%). The only exceptions occurred in Group X, where the degree of the atrophy was less (78.1%) than in all the other groups treated with COL-Ac, stress, and COL-Ac+stress (compare Group X with Groups IV–IX). Nevertheless, as judged from the final body weight and that of the intact triceps surae muscle, restraint+COL-Ac in this group (Group X) caused marked general catabolism (compare the final body weights of Group X

with Groups IV–IX). It can be assumed that the smaller degree of atrophy of the disused side in Group X was due to a technical error, because when the animals were restrained on the board with adhesive tape, the immobilized limb was not strapped down. The implications arising from this error are discussed later.

In the limbs of all the rats in Groups III–X, where increased catabolism has been described, *histological examination* showed atrophy of the triceps surae muscle of the disused limb, with the usual reduction of the gross bulk of the muscle and the dimensions of each of its constituent fibers. After 21 days' observation, no nuclear change or any degenerative process, including necrosis, was observed.

Discussion

It is evident, from the summary of our findings (see Table I), that the development of the disuse atrophy in skeletal muscle of one hind limb, which is normally caused by immobilizing the limb in a plaster cast, can be significantly aggravated by COL-Ac administration or by chronic exposure to various stressor agents. These experiments also indicate that a greater degree of atrophy results when immobilization is followed by COL-Ac plus stressors than when followed by either of these two agents alone.

It is noteworthy that, in the present experimental arrangement, the immobilization of one hind limb caused diminution in the rate of the somatic growth. It can be concluded from this finding that, in the rat, this immobilization, without damaging the tissue through compression, can act as a "neurogenic" stressor agent, thus producing general catabolism. This finding was unexpected and may have a general significance as regards the studies of disuse muscle atrophy in this species. It is probable that the resulting atrophy was not entirely due to the disuse of the muscle, but was aggravated by the "neurogenic" stress effect of the immobilization. It can be assumed that the above experimental result may have an important bearing also on the pathogenesis of muscular atrophy in general. As these experiments clearly showed, the immobilization with a plaster cast can act as a "conditioning factor" which sensitizes the muscle tissue to the catabolic effect of endogenous and exogenous corticoids, and, by selectively increasing catabolism, can significantly aggravate the development of disuse muscle atrophy. To date, in the experimental study of muscle atrophy, the significance of a possible general catabolism (i.e. stress effect), resulting from disuse, has not been considered by other workers (3, 4, 10).

The degree of development of the disuse atrophy is independent of the type of stressor used, whether alone or in combination with COL-Ac; this demonstrates the nonspecificity of the reaction. The question arises: by what mechanism does the plaster cast condition the muscle to the catabolic effect of corticoid hormones? Our recent data do not suffice to decide this question. Yet, an earlier experiment showed that an experimentally produced circulatory deficiency of an area, too slight in itself to produce any visible disturbance, can become manifest when the organism is exposed to various stressors which

create an increased production of corticoid hormones (2). Here, the limited deficiency in the arterial blood supply was a conditioning factor which selectively sensitized the muscular tissue for the catabolic effect of blood-borne corticoids. It is possible that immobilization with a plaster cast conditioned the muscular tissue in the same way as did an experimentally produced vascular deficiency, but this is an aspect of the pathogenesis of muscle atrophy which, so far, has not been studied adequately. It is generally believed that an increased blood flow occurs in the limb as a result of immobilization in a plaster cast (4, 9). This finding does not directly contradict our assumption, because an increased rate of blood flow does not necessarily indicate a greater blood volume, i.e. an increased arterial blood supply to the disused muscular tissue (12, 13).

It is well known that muscular training (even when the disused muscle is in a stretched position, as in our experimental arrangement, i.e. "isometric training"), increases the arterial blood supply in the corresponding muscular tissue (16, 17). On the other hand, we found that in the presence of an experimentally produced circulatory deficiency, a mild muscular exercise which allows for the development of compensatory vascular changes, even when combined with COL-Ac administration, produced less muscular atrophy than did any other form of stress under the same experimental conditions (2). This probably explains why the group subjected to restraint and immobilization showed less atrophy than did any other experimental group. As the immobilized limb was not strapped down during restraint, the animal used it to try to free itself. It is thought that this exercise compensated for the conditioning effect of the plaster cast.

At the present time, the question of inactivity is considered, by most authors, to be of primary importance, because this is the most common factor among various classical types of muscle atrophy (3, 6, 8, 21, 22). The hormonal production and aggravation of localized muscle atrophy offers a new approach in the study of the yet obscure pathogenesis of muscular atrophies in general.

Acknowledgments

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BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

IV. THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF THE MUSCLE[1]

J. D. Wood

Abstract

The non-protein nitrogenous constituents of muscle of migrating sockeye salmon were investigated. These constituents were found to be the same in both male and female fish and were present in approximately the same amounts in both sexes. The histidine content of the muscle in all fish decreased to one fifth of the original value during the early stages of the migratory journey and remained at the low level thereafter. Some of the other constituents changed to a smaller extent, usually increasing in the later stages of the migration. This was especially noticeable in female fish. However, the increase in the concentration of these constituents in the muscle was due to a decrease in the amount of muscle in the fish rather than to an increase in the amounts of the compounds themselves.

Introduction

Little work has been carried out on the non-protein nitrogenous constituents of migrating salmon. Greene (1) studied the muscle nitrogen extractives of king salmon, *Oncorhynchus tschawytscha*, and he found that the amino nitrogen increased during the migration and that the creatine nitrogen showed no trend either up or down. Duncan and Tarr (2) found that the non-protein nitrogen of salmon muscle did not change significantly during the migration of the fish. The individual nitrogenous constituents were investigated and the results of these studies are presented in this paper.

Materials and Methods

Muscle Samples

These were taken from the fish described by Idler and Tsuyuki (3), and were the same samples as used by Duncan and Tarr (2) for total non-protein nitrogen estimations.

Preparation of Extracts

Approximately 10 g. samples of muscle were homogenized in 3.2 volumes (v/w) absolute ethanol for 10 minutes. The resulting homogenate was kept at room temperature for 20 minutes, centrifuged at 4000 r.p.m. for 5 minutes, and the supernatant fluid decanted. The residue was resuspended for 20 minutes at room temperature in 80% ethanol equal in volume to the supernatant fluid removed. The mixture was centrifuged and the supernatant fluid collected as before. Five such successive extractions were carried out, and the supernatant fractions from these were combined and made up to 200 ml. with 80% ethanol. Aqueous extracts were prepared from the alcohol

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extracts by shaking with 3 volumes of chloroform and removing the upper aqueous layer as described by Awapara (4). Alcohol extracts were employed for the investigations unless otherwise stated.

Analyses

The α-amino nitrogen was estimated using aqueous extracts by the method of Pope and Stevens (5). Volatile-base nitrogen, trimethylamine nitrogen, and the trimethylamine oxide nitrogen were determined as described by Ronold and Jakobsen (6). Histidine was estimated by the method of Albanese *et al.* (7) with one variation; the solutions were made up to the final volume with 50% ethanol to stabilize the color. Creatinine was determined by the method of Bonsnes and Taussky (8) and creatine by that of Eggleton, Elsden, and Gough (9) as described by Ennor and Rosenberg (10).

Anserine was estimated as follows using aqueous extracts from approximately 10 g. of muscle. The diamino acids were separated from the other amino acids by means of a silicic acid column (11). The fraction containing the diamino acids also contained the anserine, which was recovered in over 90% yield. The fraction was taken down to dryness *in vacuo* and hydrolyzed in 6 *N* hydrochloric acid for 6 hours at 110° C. in a sealed tube. The solution was taken down to dryness repeatedly to remove hydrochloric acid and the residue dissolved in a small known volume of water. Samples from this, together with β-alanine standards, were chromatographed using butanol – acetic acid – water (5:1:4 v/v). The paper was dipped in a solution of 0.25% ninhydrin in acetone and the spots were developed at room temperature for 24 hours. The color density of the spots was measured in a Beckman spectrophotometer (12) and the amount of β-alanine in the samples calculated by the method of maximum density as described by Block (13). This method was applicable to the salmon muscle extracts because chromatography showed that no carnosine was present. The latter compound would otherwise have interfered since it also gives rise to β-alanine on hydrolysis. The amount of anserine in the sample was calculated from the amount of β-alanine produced by hydrolysis.

Chromatography

Two-dimensional paper chromatograms were run on appropriate amounts of muscle extract using Whatman No. 1 paper. The first solvent system was phenol–water (4:1 w/v) and the second system was collidine – 2,4-lutidine – water (1:1:1 v/w). The length of run was 25 cm. for both systems. The papers were sprayed with 0.25% ninhydrin in water-saturated butanol and the color was developed at room temperature for 24 hours.

Displacement chromatograms were obtained as follows. The nitrogenous compounds in an alcohol extract from 600 g. of salmon muscle were adsorbed on a cation exchange resin and displaced with 0.075 *N* sodium hydroxide as described by Shewan (14). The resin used was Bio Rad AG50×4 and the fractions collected were 4 ml. in volume. The compounds present in each fraction were identified (14) and the results are shown diagrammatically in Fig. 1 in order that the relative amounts of the compounds with respect to one another can readily be seen.

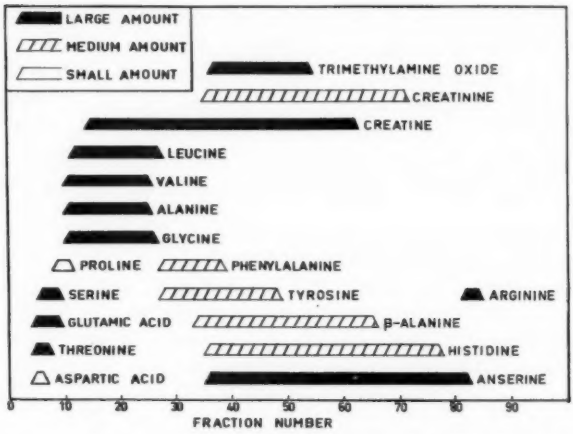

FIG. 1. Compounds displaced from cation exchange resin with sodium hydroxide.

Results and Discussion

The non-protein nitrogenous constituents of sockeye salmon muscle were investigated at three points during the migration of the fish up the Fraser River, namely at Lummi Island at the mouth of the river, at Lillooet 250 miles upstream, and at the Forfar Creek spawning grounds 753 miles upstream (3).

The muscle extracts from all fish of the same sex from each location were combined and aliquots were subjected to two-dimensional paper chromatography as described under methods. Thirteen ninhydrin spots were observed to be common to all chromatograms. Moreover the size of these spots relative to one another appeared to be constant from one chromatogram to another. In addition to the 13 spots already mentioned, a spot in the position of histidine was observed in chromatograms from both male and female fish at Lummi Island. This spot was not observed in chromatograms from fish of either sex at Lillooet or Forfar Creek.

The ninhydrin-reacting compounds were identified by their R_f values on the paper chromatograms, and further proof of their identity was obtained by employing displacement column chromatography. Figure 1 shows diagrammatically the displacement picture obtained with extracts from the muscle of male fish at Lummi Island. All the compounds identified on the two-dimensional paper chromatogram were found in the displacement chromatogram with the exception of taurine, which was not adsorbed by the resin in the column and therefore not displaced by the sodium hydroxide. The displacement picture shows, in addition, the presence of a trace of proline and the compounds creatine, creatinine, and trimethylamine oxide, which did not give a colored spot with ninhydrin. Fig. 1 also gives an indication of the relative amounts of the compounds in the muscle with respect to one another.

The fact that histidine was observed in chromatograms of muscle extracts of the fish at Lummi Island but not at other points on the river led to further investigations of that compound. Analyses showed that the histidine content of the muscle dropped sharply from Lummi Island to Lillooet and remained at a low level thereafter. These results are shown in Table I. In both male and female fish the histidine value decreased to about one fifth of the value at Lummi Island. At present no explanation can be given for this phenomenon.

TABLE I

THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF SOCKEYE SALMON MUSCLE AT DIFFERENT LOCATIONS ON THE MIGRATORY ROUTE

(All values are mg. nitrogen per 100 g. wet muscle)

Component	Sex	Lummi Island	Lillooet	Forfar Creek
α-Amino-N	M	94.8±4.7	88.4±2.3	94.4±5.5
	F	69.8±3.6	65.5±2.1	81.6±3.2
Volatile base-N	M	10.5±1.2	7.2±0.3	8.5±1.0
	F	9.3±0.7	7.9±1.2	11.4±0.6
TMO-N	M	5.4±0.3	5.4±0.8	5.3±0.4
	F	5.4±0.7	5.7±0.6	6.4±0.2
TM-N	M	<0.2	<0.2	<0.2
	F	<0.2	<0.2	<0.2
Creatinine-N	M	4.9±0.8	5.9±0.4	5.6±1.2
	F	3.7±0.4	3.7±0.3	3.1±0.5
Creatine-N	M	77.9±8.1	91.4±5.4	95.1±8.7
	F	95.5±11.8	114.4±2.8	127.0±3.6
Histidine-N	M	10.8±1.3	2.1±0.5	1.3±0.1
	F	15.1±0.8	2.7±0.4	3.1±0.1
Anserine-N*	M	24.8	31.2	30.2
	F	29.4	27.5	36.1

*These values were obtained using combined extracts from the four groups.

Table I gives the results of analyses on the salmon muscle. The values in the table are the average for four groups (3), plus or minus the standard error. These results indicate that muscle from male and female fish had the same non-protein nitrogenous constituents, and that these constituents were present in approximately the same amount in both sexes, except for creatine, which was present in somewhat larger amounts in the female, and α-amino nitrogen, which was found to a slightly greater extent in the male. The values obtained for these compounds were similar to those obtained (1) for king salmon. It is interesting to note that the dipeptide anserine was present in relatively large amounts in the muscle of both male and female fish. Little, if any, trimethylamine was present, but trimethylamine oxide was found in small quantities.

In comparison with histidine the amounts of the other constituents showed little change during the migration. Of these other compounds, creatine showed the greatest change. An increase in the level of this compound was observed during migration, especially with female fish. The α-amino nitrogen in both sexes appeared to decrease slightly from Lummi to Lillooet, and then to increase from Lillooet to Forfar Creek, but the only change which was

significant within the 1% confidence limits was that in the females between Lillooet and Forfar Creek. The volatile-base nitrogen followed a similar trend to the α-amino nitrogen.

It is seen from Table I that there was an over-all tendency for the non-protein nitrogenous constituents to increase during the journey of the female fish from Lillooet to Forfar Creek, and that this increase was much more noticeable than that observed in the male fish. However, Idler and Tsuyuki (3) have shown that the weight of the muscle in "standard fish" decreased during spawning migration, and that this loss in muscle weight was greater in the female sex. The figures obtained by Idler and Tsuyuki were employed in conjunction with the results in Table I to give an estimate of the "total amount" of non-protein constituents in the muscle. These results are given in Table II. Little or no increase in the constituents was observed from the Lillooet location to the Forfar Creek location. In other words, the increase in the concentration of the muscle constituents was balanced by the decrease in the weight of the muscle. Therefore, although the concentration of the constituents increased during the latter stages of the migratory journey, this was brought about by a decrease in the amount of flesh rather than in increase in the amount of the constituents. The results in Table II show that the main changes in the "total amounts" of the constituents during migration were small decreases in the amounts of α-amino nitrogen and volatile bases and a large decrease in histidine during the early part of the migratory journey.

TABLE II

THE AMOUNT OF NON-PROTEIN NITROGENOUS CONSTITUENTS IN THE MUSCLE OF THE "STANDARD FISH"

(All values are mg. nitrogen per fish)

Component	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
α-Amino-N	M	1391	1151	1231
	F	931	743	762
Volatile base-N	M	154	94	111
	F	124	90	106
TMO-N	M	79	70	69
	F	72	65	60
Creatinine-N	M	72	77	73
	F	50	42	29
Creatine-N	M	1143	1190	1240
	F	1274	1298	1186
Histidine-N	M	158	27	17
	F	202	31	29
Anserine-N	M	364	406	394
	F	392	312	337

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THE UTILIZATION OF ETHANOL FOR BIOSYNTHESIS IN ESCHERICHIA COLI[1]

R. P. DE LEON AND E. H. CREASER

Abstract

Radioactivity from 1-C^{14}-ethanol was incorporated into the macromolecular components of *Escherichia coli* when this organism was grown on a synthetic glucose–salts medium. No significant incorporation was observed with mammalian tissues.

Incorporation was greater in *E. coli* cells growing anaerobically than in non-growing or aerobically growing cells. The addition of sodium fumarate increased the incorporation in anaerobically growing cells approximately threefold.

Over 90% of the incorporated radioactivity was found in the lipid and protein fractions of the cell, only a small amount being detectable in the nucleic acid fraction. The distribution of radioactivity among the various cell components was such that it appeared that ethanol was utilized by the same biosynthetic pathways as acetate. No evidence could be found to support the view that ethanol carbon was incorporated into deoxyribose of the deoxyribose nucleic acid (DNA) via acetaldehyde.

Introduction

Dawes and Foster (1) have shown that ethanol disappears from cultures of *E. coli* under anaerobic conditions. It was therefore thought to be of interest to determine whether ethanol could be used for biosynthetic purposes by this organism. It is known that *E. coli* cells contain all the necessary enzymes for the conversion of ethanol to deoxyribose-5-phosphate (2, 3, 4) and so one object of this work was to assess the significance of this pathway for deoxyribose synthesis in growing cells. An alternative pathway for deoxyribose formation has been suggested by Cohen (5), who presented evidence showing that deoxyribose was derived from ribose in *E. coli*.

Experimental

Materials and Methods

In general, the experimental methods used followed closely those detailed by Roberts *et al.* (6), who studied the biosynthetic pathways in *E. coli* using a wide range of radioactive tracers.

E. coli strain B/1 was grown overnight at 37° with aeration in a synthetic salts medium ('C' medium),* pH 6.8, with glucose added to give a final concentration of 0.02 *M*. For the experiments discussed in this work, amounts of such a culture were diluted with fresh medium containing the appropriate

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Contribution from the McGill – Montreal General Hospital Research Institute, Montreal, Quebec. This investigation was supported by grants from the Committee on Problems of Alcohol, National Academy of Sciences – National Research Council and from the Rockefeller Foundation.

*C medium: 2 g. NH_4Cl, 6 g. Na_2HPO_4, 3 g. KH_2PO_4, 3 g. NaCl, 0.01 g. Mg as $MgCl_2$, 0.026 g. S as Na_2SO_4, and 1000 ml. distilled water.

supplements, so that the final concentration of cells was 3×10^8/ml. A calibration curve was constructed to give the relationship between the number of cells per milliliter and the optical density (O.D.) of the bacterial suspension when the latter was measured in a Fisher colorimeter at 650 mμ.

After the experimental incubation periods, the growth of the cells was estimated by measurement of the increase in O.D. of the culture. The cells were subsequently precipitated by adding trichloroacetic acid (TCA) to the medium to give a final concentration of 5%. After it was cooled at 4° for 1 hour, the TCA precipitate was removed by means of a membrane filter (7) and its radioactivity assayed using a Geiger–Muller mica end-window tube with associated counting equipment. This measured total incorporation into the macromolecular components of the cell.

In experiments where more specific localization of the incorporated radioactivity was desired, the cells were removed from their medium by centrifugation, washed by suspension in fresh C medium, and recentrifugation, then precipitated with 5 ml. of 5% TCA. After an hour at 4°, the TCA precipitate was sedimented, washed by suspension in 5 ml. of 5% TCA and recentrifugation, then suspended in 80% ethanol. The two TCA supernatants were combined and an aliquot of the solution was plated and counted. This fraction contained the low molecular weight metabolic intermediates.

The TCA-insoluble material was extracted successively with 5-ml. portions of 80% ethanol and a 1 : 1 mixture of 95% ethanol and ether at 50°, for 30 and 15 minutes respectively. This procedure brought into solution the lipids and alcohol-soluble proteins.

The insoluble material left from this treatment was then dissolved in 1 *M* NaOH and left to stand at room temperature for 18 hours (8). TCA was then added to this solution to give a final concentration of 5%. Precipitated material was centrifuged and washed by suspension in 5 ml. of 5% TCA and recentrifugation. The two TCA supernatants contained the hydrolysis products of ribose nucleic acid (RNA).

The precipitated material was extracted with 5 ml. of 5% TCA at 100° for 30 minutes, centrifuged, and washed with 5 ml. of 5% TCA. The soluble material contained the hydrolysis products of DNA, and the insoluble material contained the protein.

In certain cases, the protein and nucleic acid fractions were dried and then hydrolyzed in 1 ml. of 6 *N* HCl at 100° for 18 and 2 hours respectively. The hydrolyzates were subjected to two-dimensional paper chromatography. The solvents for the protein hydrolyzates were secondary butyl alcohol – 90% formic acid – water, and phenol–ammonia (see ref. 6, pp. 36–41) for the first and second dimension solvents respectively. For the nucleic acid hydrolyzates, the solvents used were isopropanol–HCl and butanol–ammonia (9) for the first and second dimension solvents respectively. Radioactive areas on the chromatograms were located by radioautography.

1-C^{14}-Ethanol was obtained from Atomic Energy of Canada Ltd. and from Merck and Co. Inc.

Results

Preliminary experiments using standard manometric techniques showed that ethanol was used as a substrate for respiration by *E. coli* when present in concentrations not greater than 2 *M*, higher concentrations being inhibitory. It was found that reproducible results on the incorporation of radioactive ethanol could be obtained only when a closed system was used for the incubation procedure.

Incorporation of 1-C^{14}-Ethanol by E. coli

An experiment was performed to provide information on some of the conditions affecting the incorporation of ethanol into the macromolecular components of *E. coli* and the results obtained are shown in Table I. It can be seen that, in the absence of glucose, there was little growth or incorporation of radioactivity by the cell. When glucose was added, growth occurred and incorporation took place, the observed amount of radioactivity incorporated being the same under aerobic and anaerobic conditions. The further addition of sodium fumarate stimulated growth (10), and greatly increased incorporation under anaerobic conditions. Sodium fumarate alone was less effective than glucose alone. Thus, the greatest incorporation was observed under anaerobic conditions at 37° in the presence of 0.02 *M* glucose and 0.05 *M* sodium fumarate. These conditions were therefore adopted as standard for all subsequent experiments reported here.

TABLE I

INCORPORATION OF RADIOACTIVITY FROM 1-C^{14}-ETHANOL BY *E. coli*

Additions	Increase in O.D.		Incorporation into TCA insolubles, c.p.m.*	
	Aerobic (air)	Anaerobic (N_2/CO_2)	Aerobic (air)	Anaerobic (N_2/CO_2)
Nil	0.05	0.04	8	5
Glucose	0.45	0.20	88	88
Glucose and fumarate	0.60	0.36	72	247
Fumarate	0.29	0.04	52	12

*c.p.m.—counts per minute. All incubation mixtures initially contained 3×10^8 cells per milliliter (O.D.=0.10) suspended in 2 ml. of C medium with 5 μc. 1-C^{14}-ethanol per milliliter and unlabelled carrier ethanol added to give a final ethanol concentration of 0.1 *M*. Glucose and sodium fumarate were added where stated, to give final concentrations of 0.02 and 0.05 *M* respectively. Incubations were carried out at 37° for 2½ hours. After the incubation periods, the cells were treated as described in the text.

Figure 1 shows that the amount of radioactivity incorporated under these conditions was increased by raising the ethanol concentration until maximum incorporation was reached at an ethanol concentration of 0.15 *M*, higher concentrations being inhibitory.

The results shown in Table I indicated that the uptake of radioactivity was dependent on growth of the organism; this is clearly shown in Fig. 2, which shows that the two processes are directly proportional.

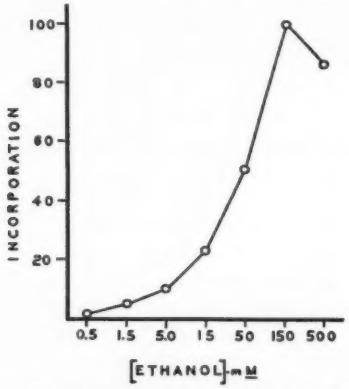

FIG. 1. Effect of ethanol concentration on incorporation of radioactivity from 1-C^{14}-ethanol into the macromolecular components of *E. coli*. Each incubation had 5 μc./ml. of 1-C^{14}-ethanol plus unlabelled ethanol added to the concentration shown. Cells were grown in 2 ml. C medium for 4 hours with 0.02 *M* glucose and 0.05 *M* sodium fumarate under an atmosphere of 95% N_2/5% CO_2 at 37°. Incorporation is plotted as percentage of the maximum observed values.

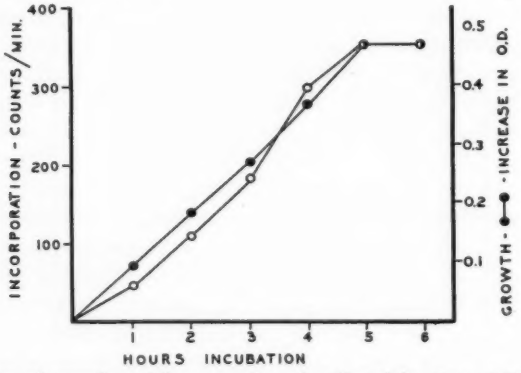

FIG. 2. Rate of growth and incorporation of radioactivity from 1-C^{14}-ethanol into the macromolecular components of *E. coli*. Ethanol concentration was 0.15 *M*, other conditions as in Fig. 1.

Figure 3 indicates that the incorporation of radioactivity from 1-C^{14}-ethanol into the protein fraction of *E. coli* is reduced by the addition of acetaldehyde to the incubation mixture. Incorporation is abolished when 0.015 *M* acetaldehyde is present in addition to 0.15 *M* ethanol.

Several experiments were performed with mammalian tissues including brain, using the same incubation conditions which had been found to be effective in promoting the incorporation of radioactivity from 1-C^{14}-ethanol into the macromolecular components of *E. coli*. No significant incorporation was observed and so these experiments were not continued.

Distribution of Incorporated Radioactivity

A detailed study of the incorporation of radioactivity from ethanol into the different cell components was next undertaken. For these experiments,

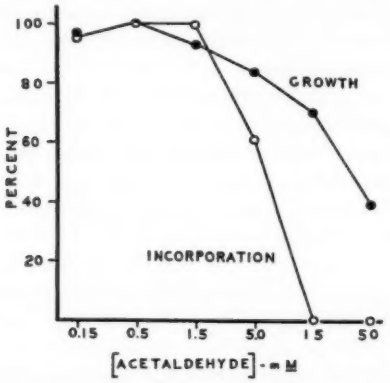

FIG. 3. Effect of acetaldehyde concentration on growth and incorporation of radioactivity from 1-C^{14}-ethanol into the protein fraction of *E. coli*. Acetaldehyde concentration was as shown, other conditions as in Fig. 1. Growth and incorporation plotted as percentage of control which contained no acetaldehyde.

the bacteria were incubated anaerobically at 37° in C medium containing 0.02 *M* glucose, 0.05 *M* sodium fumarate, 0.15 *M* ethanol, and 10 μc. 1-C^{14}-ethanol/ml. A parallel experiment was performed which differed only in that the ethanol was replaced by 0.15 *M* sodium acetate and 10 μc. of 1-C^{14}-sodium acetate/ml. of medium. After incubation, the radioactivity incorporated into the different cell fractions was measured as described above. The results obtained from duplicate experiments utilizing the above tracers are shown in Table II. It can be seen that the bulk of the radioactivity was located in the lipid and protein fractions. The RNA and DNA fractions were separated in one experiment and it was found that where ethanol was used as a tracer, approximately 3% of the total incorporated radioactivity was in the RNA portion and 2% in the DNA. With radioactive acetate as tracer material, about 2% was found in the RNA fraction and 1% in the DNA fraction.

TABLE II

INCORPORATION OF RADIOACTIVITY FROM 1-C^{14}-ETHANOL AND 1-C^{14}-SODIUM ACETATE BY *E. coli*

Fraction	Percentage activity			
	1-C^{14}-Na acetate		1-C^{14}-Ethanol	
	I	II	I	II
Metabolic intermediates	12.8	11.5	11.8	12.4
Lipids and alcohol-soluble proteins	45.0	46.5	38.2	39.3
Total nucleic acids	3.3	3.5	5.6	5.0
Residual protein	35.2	32.8	37.8	34.7
Percentage recovery	96.3	94.3	93.4	91.4

NOTE: *E. coli* (3×10^8 cells/ml.) incubated at 37° for 4 hours under an atmosphere of 95% N_2/5% CO_2 in 10 ml. C medium with additions as described in the text. In one case, 10 μc./ml. 1-C^{14}-Na acetate was present, in the other, 10 μc./ml. 1-C^{14}-ethanol. After incubation, cells were fractionated as described in the text.

The specific activities of these fractions were so low that the differences in the incorporation in the RNA and DNA from the acetate and ethanol experiments were not considered significant.

The protein and nucleic acid fractions were hydrolyzed and subjected to two-dimensional paper chromatography. Radioautographs of the protein fraction showed that the following amino acids were radioactive: proline, arginine, lysine, glutamic and aspartic acids, and leucine–isoleucine. This distribution was the same whether ethanol or acetate had been used as the radioactive tracer.

It was not possible to determine completely the distribution of radioactivity in the nucleic acid fraction of the cells as insufficient radioactivity had been incorporated. Traces of activity from both tracers were present in several of the bases.

In parallel experiments, attempts were made to isolate any radioactive deoxyribose that may have been present in the DNA fraction of the cells. Hydrolysis of the DNA by either chemical (11) or enzymic (12) methods followed by paper chromatography of the hydrolysis products (13) failed to show the presence of any radioactive deoxyribose.

It was found that when the glucose in the medium was replaced by 1-C^{14}-glucose, the extent of incorporation of radioactivity from glucose into the nucleic acid fraction was not detectably influenced by the presence or absence of ethanol in the medium. However, the incorporation into the protein and lipid fractions was reduced by 35–45% when 0.15 *M* ethanol was present as shown in Table III.

TABLE III

EFFECT OF ALCOHOL AND SODIUM ACETATE ON THE INCORPORATION OF RADIOACTIVITY FROM 1-C^{14}-GLUCOSE BY *E. coli*

Fraction	Incorporation into fractions (c.p.m./increase in O.D.)		
	Control	In presence of 0.15 *M* ethanol	In presence of 0.15 *M* acetate
Metabolic intermediates	109,500	100,100	68,000
Lipids and alcohol-soluble proteins	63,630	35,250	32,130
Total nucleic acid	91,200	91,500	78,700
Residual protein	92,200	60,000	67,800
Total	356,530	286,850	246,630

NOTE: *E. coli* (3×10^8 cells/ml.) incubated at 37° for 4 hours under an atmosphere of 95% N_2/5% CO_2 in 10 ml. C medium with 5 μc./ml. of 1-C^{14}-glucose. Final glucose concentration in all cases was 0.02 *M*. Other conditions as described in the text. After incubation, cells were fractionated as previously described. Activity expressed per increase in optical density.

Discussion

The work of Roberts *et al.* (see ref. 6) has shown that it is possible to obtain information on the metabolic pathways of radioactive materials by adding suspected intermediates to the system and then determining the radioactivity of the products. If the suspected intermediate is indeed on the route from

the radioactive precursor to the isolated product, the latter does not become radioactive when suitable amounts of the intermediate are present, presumably through dilution of the radioactivity. This approach has been called 'isotopic competition' and we have applied it to the effect of acetaldehyde on the incorporation of radioactivity from 1-C^{14}-ethanol into the protein fraction of *E. coli*. The total suppression of ethanol incorporation by 1/10th its concentration of acetaldehyde supports the view that this latter compound is on the pathway of ethanol utilization. The subsequent fate of the ethanol carbon can be deduced from its distribution, which is very similar to that of acetate.

Incorporation of carbon from ethanol occurs mainly into the protein and lipid fractions of the cell, and very little into the nucleic acid. The incorporation into DNA is so low that it is not feasible to consider ethanol as a major precursor of DNA deoxyribose, nor was it possible to find any radioactivity in the deoxyribose fraction. It is possible to calculate from the observed increases in cell mass during incubation that if ethanol, via acetaldehyde, was the source of two of the five deoxyribose carbon atoms, the incorporation of radioactivity into deoxyribose of DNA should have been at least 10 times greater than that found in the total DNA fraction in the experiments quoted above. A compatible result was obtained when it was found that ethanol did not reduce the amount of radioactivity incorporated into the nucleic acid fraction from 1-C^{14}-glucose, whereas there was a reduction of the amount incorporated into the protein and lipid fractions.

Thus, the results indicate that ethanol is used for biosynthetic purposes in *E. coli* by the same pathways as is acetate. No evidence was obtained to suggest that ethanol, and hence acetaldehyde, was used for nucleic acid deoxyribose synthesis in growing *E. coli*.

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OBSERVATIONS ON THE UPTAKE OF RADIOSULPHATE BY THE GASTRIC TISSUE AND GASTRIC SECRETION IN HISTAMINE-TREATED GUINEA PIGS[1]

K. KOWALEWSKI AND H. T. G. WILLIAMS

Abstract

The effect of histamine on the uptake of radiosulphate by gastric tissue and gastric juice in guinea pigs was investigated. Radioactivity was recorded 4 hours after the administration of S^{35}-labelled sulphate to normal controls and to histamine-treated animals.

The radioactivity of fractions considered to contain sulphated mucopolysaccharides was also investigated.

The uptake of radiosulphate by gastric tissue and juice was significantly higher in histamine-treated animals than in control guinea pigs.

The S^{35} uptake by gastric sulphomucopolysaccharides was considered to represent the concentration of isotope by mucous cells and its secretion. This selective uptake was also markedly effected by histamine.

Histamine may be a stimulant of the gastric mucous cells under the experimental conditions described.

Introduction

When S^{35}-labelled sulphate is administered to animals it may follow several metabolic pathways. Most of the sulphate ion is excreted as inorganic and ethereal sulphate; a small fraction is retained in sulphated mucopolysaccharides and a trace is bound to sulphur-containing amino acids (1). Certain tissues and organs consistently take up large amounts of S^{35}. The selective uptake of radiosulphate in cartilage, bones, skin, and healing wounds is currently explained by the utilization of sulphate ions in the synthesis of certain mucopolysaccharides of connective tissue (2, 3).

The mucous glands of gastrointestinal tract may also utilize the labelled sulphate and this has been found mostly in the form of chondroitin or mucoitin sulphate. It has been shown that not only is radiosulphate taken up selectively by gastric or intestinal mucosa but also that the mucus secreted contains a marked amount of this isotope (4, 5).

Gastric mucus has been previously studied by various physical and chemical methods, but the knowledge of this mucoprotein is still very limited. Especially controversial is the problem of the mechanism of stimulation of mucus-secreting cells.

Histamine for example is not considered by Babkin as a stimulant of mucus secretion (6). The classical opinion that parasympathetic stimuli induce the secretion of mucus (7, 8) is not generally accepted (9, 10).

In previous work on Heidenhein pouch secretion in dogs, we were able to show that the uptake of radiosulphate by the pouch juice of histamine-treated dogs was significantly higher than in control dogs (11). In this study only total radioactivity of juice was determined. The assumption that this may represent the increased posthistaminic production of labelled sulphomucopolysaccharides needs further experimental evidence.

[1]Manuscript received November 4, 1957.
Contribution from the McEachern Cancer Research Laboratory and the Department of Surgery, University of Alberta, Edmonton, Alberta.

The present report concerns the effect of histamine on the uptake of labelled sulphate by gastric tissue and gastric juice in guinea pigs. An attempt was made to determine also the S^{35}-sulphated mucosubstance by appropriate fractionation of samples.

Experimental

Seventy male guinea pigs, fed on commercial pellets and vegetables, and having a weight range of 689 to 800 g., were used. They were divided into two equal groups. Forty-eight hours before the administration of radiosulphur the animals were kept in separate cages and allowed water only.

Treatment

Radiosulphur was given subcutaneously in doses of 400 μc. per kg. of body weight. The dose of commercial isotope ($H_2S^{35}O_4$, Atomic Energy of Canada) was dissolved in 2 ml. of distilled water together with 16 mg. of sodium sulphate (12). Histamine dihydrochloride (Roche) was administered intramuscularly in a dosage of 75 mg. per kg. of body weight, 30 minutes after the subcutaneous injection of a protective dose of antihistamine promethazine HCl (Phenergan, Poulenc) in a dosage of 30 mg. per kg. of body weight (13). This dosage of histamine is sufficient to produce acute lesions of gastric mucosa in guinea pigs (13, 14). Isotope was given 5 minutes after the injection of histamine. This treatment was given to 35 animals. The other group of 35 guinea pigs served as normal controls and received radiosulphate alone.

Sampling

All animals were anesthetized 4 hours after the dosage of isotope. The stomach was removed with its content, the juice collected into a beaker, and the mucosa rinsed with 10 ml. of distilled water, the washings being added to the gastric juice. The stomach was opened along the great curvature and examined for mucosal lesions, under the dissecting microscope (14). It was then washed under running distilled water to remove the blood and placed in a container for digestion.

In some animals blood was collected in heparinized tubes from the inferior *vena cava* and plasma separated.

In some animals the right humerus was also dissected and prepared for the digestion. Bone was studied in order to compare the gastric S^{35} uptake with that of a tissue (bone) having specifically high sulphate-fixing capacity (12).

Measurement of Radioactivity

Two general procedures were used in this experiment. Forty animals were studied for total radioactivity of gastric tissue, gastric juice, and bones. In the remaining 30 guinea pigs an attempt was made to isolate sulphated mucopolysaccharides in radioactive samples of gastric tissue, gastric secretion, and plasma.

For the determination of total radioactivity the bones, stomachs, and samples of juices were treated by the nitric–perchloric acid wet-digestion procedure described in detail in our previous report (12). The stomachs studied for the radioactive mucopolysaccharides were minced and extracted for 48 hours with 95% ethanol (15). Sulphated mucopolysaccharides were then isolated from tissue by the extraction with KOH (16). Samples of minced gastric tissue, dried after alcohol extraction, were homogenized individually in 50 ml. of 2% aqueous KOH in a Waring blendor for 20 minutes. The blendor was then washed with an extra 50 ml. of KOH and the total suspension was shaken at room temperature for 24 hours. After the KOH extract was centrifuged, it was deproteinized with 4:1 mixture of chloroform and amyl alcohol (17).

Final aqueous solution containing sulphated mucopolysaccharides was dried by heating and the barium sulphate was isolated following the nitric–perchloric acid procedure (12). The radioactivity of the alcohol and chloroform – amyl alcohol extracts was determined by the same method.

Total gastric juice and a 2-ml. sample of plasma was dialyzed against distilled water (18). It is known that non-dialyzable sulphated mucosubstances are retained by the membrane and the diffusible radioactivity found in water is due in major part to $S^{35}O_4^{2-}$ and may be removed as barium sulphate.

In this experiment both the content of cellophane bag and the dialysis water were studied for radioactivity, using the barium sulphate precipitation method mentioned above (12).

The final precipitate of radioactive barium sulphate was transferred to planchettes and the counting carried out by using a thin mica window Tracerlab G-M tube. A Tracerlab Superscaler S.C. 18A was used for recording.

The results were recorded in counts per minute (c.p.m.) and some of them presented as a percentage of the dose of S^{35} injected into animals.

The radioactivity of various fractions of analyzed samples was expressed as a percentage of total radioactivity of a sample.

Results

All histamine-treated animals presented microscopically obvious gastric lesions as described previously (14). Volumes of gastric juice recorded in control and histamine-treated guinea pigs were 7 ± 1.7 ml. and 14 ± 7 ml.; body weights were 729 ± 81 g. and 731 ± 71 g. respectively.

Table I and Fig. 1 present data on recorded total radioactivity of tissue and gastric secretion. It may be noted that the difference in the percentage of uptake between normal control and histamine-treated animals was significant. The concentration of radiosulphate in the gastric juice secreted by histamine-treated guinea pigs was higher than that of normal animals. There was no difference in the S^{35} uptake by the humeri.

TABLE I

TOTAL RADIOACTIVITY OF GASTRIC TISSUE, GASTRIC JUICE, AND OF A BONE IN NORMAL CONTROLS AND IN HISTAMINE-TREATED GUINEA PIGS INJECTED WITH RADIOSULPHATE (S^{35})

(Radioactivity is expressed as percentage of injected dose)

Samples	Values	Controls	Histamine-treated
Number		20	20
Total gastric tissue	Mean	0.33	0.72
	S.D.	±0.08	±0.11
	Range	0.19–0.46	0.62–0.94
Total gastric juice	Mean	0.06	0.69
	S.D.	±0.02	±0.19
	Range	0.03–0.10	0.51–1.00
1 ml. of gastric juice	Mean	0.011	0.055
	S.D.	±0.001	±0.002
	Range	0.006–0.020	0.028–0.107
Right humerus	Mean	0.21	0.24
	S.D.	±0.04	±0.07
	Range	0.15–0.30	0.10–0.36

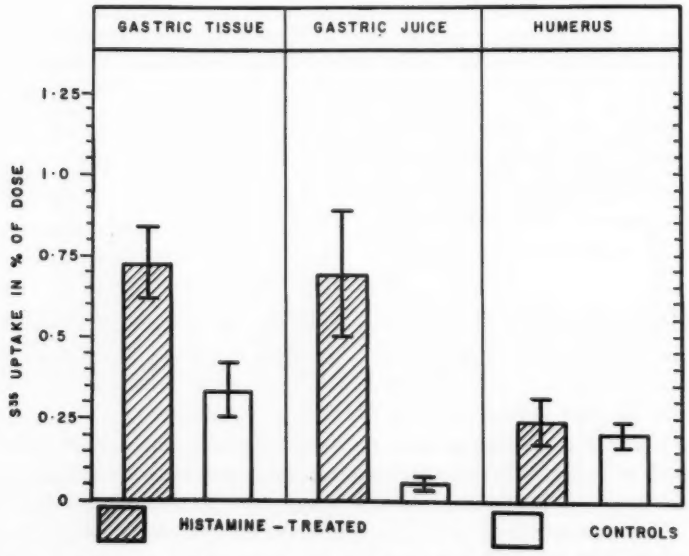

FIG. 1. Effect of histamine on total radioactivity of gastric tissue, gastric secretion, and bones of guinea pigs. Mean values and S.D. (vertical lines).

Tables II and III summarize data on fractionation of radioactive samples. It may be noted that the fractions considered to contain sulphated mucopolysaccharides were more radioactive in histamine-treated animals than in controls. Both total radioactivity and relative content of radioactive mucopolysaccharides were increased in plasma, gastric juice, and gastric tissue of guinea pigs which received histamine.

TABLE II

TOTAL RADIOACTIVITY OF GASTRIC TISSUE AND PERCENTAGE OF RADIOSULPHATE TAKEN UP BY VARIOUS FRACTIONS OF GASTRIC TISSUES IN NORMAL CONTROLS AND IN HISTAMINE-TREATED GUINEA PIGS

Sample	Values	Controls	Histamine-treated
Number		15	15
Total gastric tissue, c.p.m. ×1000	Mean	48.4	80.8
	S.D.	±7.7	±9.5
	Range	35.0–51.0	68.0–88.2
Ethanol fraction, %	Mean	28.2	18.3
	S.D.	±4.1	±3.8
	Range	24.5–33.8	14.5–21.0
KOH fraction, %	Mean	70.1	80.2
	S.D.	±2.5	±2.2
	Range	64.0–74.2	77.1–84.0
Chloroform – amyl alcohol fraction, %	Mean	<2.0	<2.0

TABLE III

TOTAL RADIOACTIVITY OF PLASMA AND GASTRIC JUICE (C.P.M.) AND PERCENTAGE OF RADIOACTIVITY RETAINED IN DIALYZED SAMPLES, IN NORMAL CONTROLS, AND IN HISTAMINE-TREATED GUINEA PIGS, INJECTED WITH RADIOSULPHATE (S^{35})

Samples	Values	Controls	Histamine-treated
Number		15	15
Total plasma, c.p.m./ml. ×1000	Mean	16.0	32.1
	S.D.	±1.9	±5.4
	Range	11.0–18.1	22.1–36.0
% of radioactivity in dialyzed plasma	Mean	18.4	24.5
	S.D.	±3.7	±2.8
	Range	12.0–19.3	22.1–27.4
Total gastric juice, c.p.m. ×1000	Mean	34.8	150.6
	S.D.	±9.5	±75.7
	Range	12.0–42.0	91.0–256.0
% of radioactivity in dialyzed gastric juice	Mean	14.5	28.0
	S.D.	±5.2	±4.9
	Range	6.2–21.6	24.1–37.1

Comment

It is known that only certain cells of the stomach take up S^{35} from the injected radiosulphate and incorporate it in the produced mucin. The fixation of isotope in the cells begins soon after the administration of S^{35} and the amount of maximal cellular radioactivity remains practically unchanged between 1 and 6 hours, falling rapidly in the next few hours (4, 19). The 4-hour period chosen in this experiment allowed the study of fixed sulphate in optimal conditions.

The separation of soluble sulphate from insoluble ("fixed") tissue sulphate and further differentiation procedures used in this study permitted the evaluation of the effect of histamine on S^{35} uptake with more precision than was done in our study on dogs (11).

Our previous assumption that histamine may stimulate the secretion of radioactive mucus (11) seems to find the experimental verification in the present study.

The increased uptake of radiosulphate by gastric tissue of histamine-treated guinea pigs apparently reflects the changes in sulphomucopolysaccharides in the gastric wall. The exact distribution of the radioactivity in various elements of gastric mucosa could be studied further by autoradiography (4).

The fact that histamine does not influence the S^{35} uptake by humeri is significant. It means that histamine does not increase the concentration of S^{35} in all tissues with sulphate-binding capacity.

The marked retention of radioactivity in plasma of histamine-treated animals is also interesting. It is apparent that histamine may play a role in mobilization of blood and tissue basophiles and is closely related to the function of connective tissue mast cells. These cells selectively take up radiosulphate (20, 21, 22, 23, 24, 25). That these cells contribute to the posthistaminic radioactivity of plasma and gastric tissue cannot, however, be concluded without radioautography.

Under the described experimental conditions histamine causes a definite alteration in the uptake of labelled sulphate by gastric tissue and gastric secretion. This alteration chiefly concerns the radioactivity of sulphated mucopolysaccharides.

Acknowledgments

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THE PRESENCE OF A MITOSIS INHIBITOR IN THE SERUM AND LIVER OF ADULT RATS[1]

H. F. Stich and M. L. Florian

Abstract

The influence of serum and tissue homogenates on the mitotic rate of regenerating liver was tested. The following fractions were injected into Sprague–Dawley rats 24 hours after partial hepatectomy: (*a*) serum from normal 290–340 g. rats; (*b*) serum from rats 24 or 72 hours following partial hepatectomy; (*c*) liver homogenates from normal 290–340 g. rats; (*d*) regenerating liver homogenates (24 hours after partial hepatectomy); and, as controls, (*e*) brain homogenates representing non-mitotic tissues; (*f*) testes homogenates representing mitotically active tissues. Serum and liver from adult animals inhibit the onset of mitosis. Serum and regenerating liver from partially hepatectomized rats, as well as heterologous tissue, show no retarding effect.

The results suggest the presence of an organ-specific inhibitor of mitosis in the serum and liver of adult animals.

Introduction

Our knowledge of factors regulating mitotic activity in mammalian tissues is still very limited, as is shown in the reviews by Hughes (1) and Swann (2). Hormones (3, 4, 5), nutritive compounds (6, 7), and energy rich substances (8, 9) have been found to influence the mitotic rate in various tissues. These factors seem to enhance or retard cell division, but can hardly be considered responsible for tissue-specific mitotic rates during normal, regenerative, or pathological growth.

The present investigation was carried out to gain some basic information about the factors which induce or inhibit mitosis in regenerating tissue. The liver of the rat was chosen as a suitable test organ, as, by means of partial hepatectomy, a tissue high in mitotic activity can be obtained and the regenerative processes are well known (10). Furthermore, results from experiments with parabiotic rats (11, 12), and from injections of serum from partially hepatectomized rats (13), indicated the presence of a mitosis trigger compound in the blood. Continuing on this line, an attempt was made to detect the presence of a mitosis inducer or inhibitor in the serum, as well as in homologous and heterologous tissues, which might lead to a better understanding of the mechanism regulating mitosis.

Materials and Methods

The sera and tissue samples were obtained from male rats of the Sprague–Dawley strain weighing 290–340 g., referred to as "adult rats". Animals 240–260 g. in weight were used for the partial hepatectomies, performed according to the technique of Higgins and Anderson (14). Removal of the median and left lateral lobes of the liver produced approximately a 70% loss in liver tissue.

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Contribution from the Saskatchewan Research Unit, National Cancer Institute of Canada, and Department of Cancer Research, University of Saskatchewan, Saskatoon, Sask.

The procedure for all experiments, unless otherwise specified, was as follows: The sera and tissue homogenates were injected intraperitoneally into partially hepatectomized rats 30 to 32 hours after operation. The test animals were killed 15 to 16 hours after the injections and liver tissue for microscopic analysis was fixed. To avoid any misleading interference resulting from the periodicity of the mitotic rate (15) a rigid timing schedule was followed: the partial hepatectomies were performed at 10–12 a.m., the different samples were injected at 5–6 p.m. on the following day, and the tissue was fixed the next morning at 10 a.m. The temperature, which influences the mitotic rate in mammals to a certain degree, was kept constant at 24 ± 1° C. in the animal and work rooms.

The tissue homogenates were prepared by grinding the tissue with an equal volume of sterile 0.85% saline in a glass Ten Broech homogenizer until the suspension would pass easily through a No. 21 needle. The homogenizer was kept cool in an ice bath during the procedure, which lasted 5–10 minutes. The amount of homogenate injected was 0.7 g. per 100 g. body weight.

For microscopic examination small pieces of liver were fixed in 3:1 ethyl alcohol – acetic acid, processed through the ethyl alcohol–butanol–benzene–paraffin schedule, sectioned at 7 μ, and stained with an aqueous, unbuffered 0.5% solution of toluidine blue. A micrometer disk was placed in the eye piece of the microscope and the number of mitoses present in the field was counted. A total of 200 fields per rat were analyzed if the mitotic rate was lower than 1%, and 150 fields were examined if more than 1% of the cells were in cell division. The number of nuclei of hepatic cells in every 10th field was counted. The mitotic rate is expressed as the number of mitoses per 1000 cells. Liver cells in division are larger than in the interphase and therefore appear on more than one serial section; to avoid duplication of counts every second section was used for analysis. Only definite prophase and early telophase nuclei were included in the counts.

Results

1. *The Inhibitory Effect of Sera*

Serum from adult rats decreased the mitotic rate in regenerating liver tissue of partially hepatectomized animals (Table I). Incomplete inhibition might be the result of too small an amount (4 ml.) of serum injected. It might also be possible that the time between application and the collecting of tissue samples (15–16 hours) was too short. Cells once in preparation for mitosis might have completed the cell division under the influence of the serum. The lower number of prophases in the inhibited liver tissue, as compared with the other samples in Table I, suggests that the serum is more effective in blocking the initiation of mitosis than in blocking mitosis which has already begun. A similar phenomenon was noted in rats injected with liver homogenates of adult animals (Table III).

Contrary to the inhibitory effect of serum from adult normal rats the serum from partially hepatectomized animals increased the rate of cell division. The means of the mitotic indices were significantly greater than those

of partially hepatectomized animals. The distribution of pro-, meta-, ana-, and telo-phases of mitosis in the rats injected with serum from partially hepatectomized animals followed the normal pattern (Table I). The presence of anaphases and telophases indicates that the effect of these sera is quite unlike that of colchicine, which blocks mitosis in the metaphase. Thus the stimulating action of the serum can not be explained by a mere prolongation of the time of a mitotic stage or accumulation of metaphases. It appears that in this case the serum enhances the mitotic rate, a result which was also obtained by Friedrich-Freksa and Zaki (13).

The inhibitory effect of the serum from normal rats practically disappeared 40 hours after a single injection (Table II). An accurate timing of the duration of the inhibition is complicated by the fact that the mitotic rate in the liver is characterized by a daily periodicity. It might well be that the suppressing effect of the serum is already compensated at 23 hours after the serum injection, but this can not be determined since at this time the mitotic rate is normally at the low point (15). The decline at this time in the effectiveness of the serum injected is indicated by the relative increase in the number of prophases. Thus the inhibitory action lasts from 15 to 23 hours. The duration of inhibition in the present experiments corresponds to the time of onset of mitosis after partial hepatectomy, between 20 and 24 hours (10).

TABLE I

THE INFLUENCE OF A SINGLE INJECTION OF SERUM ON THE MITOTIC RATE OF REGENERATING LIVER TISSUE

Types	Amount of serum injected*	No. of rats	No. of cells counted per rat	Σ Mitosis per 1000 cells		Percentage			
				Average	Range	Pro	Meta	Ana	Telo
Normal adult	0	10	25,000	0.02	—	—	—	—	—
Part. hepatectomy	0	8	18,000	31	27–35	23	42	5	30
Part. hepatectomy and:									
(*a*) serum from normal rats	1.5	5	25,000	3	1–4	8	54	4	34
(*b*) serum from part. hepatectomy									
24 hr.	1.5	4	18,000	43	40–46	24	39	5	32
72 hr.	1.5	4	18,000	62	59–64	29	38	3	30

*Expressed in ml. per 100 g.

TABLE II

THE MITOTIC INDEX AT VARIOUS TIME INTERVALS AFTER A SINGLE INJECTION OF SERUM FROM ADULT RATS

Types	No. of rats	No. of cells counted per rat	Σ Mitosis per 1000 cells		Percentage			
			Average	Range	Pro	Meta	Ana	Telo
Part. hepatectomy	8	18,000	31	27–35	23	42	5	30
Part. hepatectomy + serum, time after injection and time of day								
16 hr., 10 a.m.	5	25,000	3	1–4	8	54	4	34
23 hr., 5 p.m.	4	18,000	7	4–11	21	47	4	28
40 hr., 10 a.m.	4	18,000	25	18–31	30	46	3	21

2. The Inhibitory Effect of Tissue Homogenates

Liver homogenate from adult rats strongly inhibited the mitosis of the regenerating liver, whereas suspensions of regenerating liver showed only a slight suppression of the number of mitoses (Table III). Testes and brain were used to study the influence of heterologous tissue on the cell division of hepatic cells. Testes were selected as an example of a tissue rich in mitosis, and brain as a mitotically inactive tissue. Injection of tissue homogenates of both these organs had no significant effect on the mitotic rate of liver, as is seen in Table III. The injected homogenates frequently induced an excessive fluid accumulation in the abdominal cavity, but the liver of the injected rats show no cellular infiltration.

TABLE III

THE INFLUENCE OF SINGLE INJECTIONS OF HOMOLOGOUS AND HETEROLOGOUS TISSUE BREIS ON THE MITOTIC RATE OF REGENERATING LIVER

Types	Amount of brei injected*	No. of rats	No. of cells counted per rat	Σ Mitosis per 1000 cells		Percentage			
				Average	Range	Pro	Meta	Ana	Telo
Normal adult	0	10	25,000	0.02	—	—	—	—	—
Part. hepatectomy	0	8	18,000	31	27–35	23	42	5	30
Part. hepatectomy and:									
(*a*) normal liver brei	0.7	8	25,000	3	1–6	11	57	4	28
(*b*) regenerating liver brei	0.7	8	18,000	29	25–32	30	37	4	29
(*c*) testes brei	0.7	4	18,000	26	25–28	25	36	5	34
(*d*) brain brei	0.7	4	18,000	28	26–29	28	34	5	33

*Expressed in g. wet weight of liver tissue per 100 g. body weight.

Discussion

The experiments presented here suggest that in the serum of adult rats one or more factors are present which inhibit cell division in hepatic tissues. This inhibitory effect of the serum ceases, however, within 24 hours if parts of the liver are removed. If the effect of the sera on liver mitosis is compared with the effect of injected homogenates of normal adult and regenerating liver a striking similarity is evident. Only the adult liver homogenates decrease cell division, whereas the regenerating liver is free of any inhibitory effect. Injected heterologous tissue, brain and testes, are without any significant influence on the mitotic rate of hepatic cells.

These results suggest that an organ-specific mitotic inhibitor is present in the serum and in the liver. The experiments of Ebert (16) and Rose (17) on embryonic tissue support this idea. Both workers found that extracts of organs inhibited the development of the homologous organ growing in the extracts. These findings are in agreement with the experiment of Glinos and Gey (18), who found that the serum of fully grown rats inhibited the outgrowth of liver explants in tissue culture.

A single injection of adult serum exhibits the inhibiting effect for approximately one day. This suggests that the inhibitor(s) is rapidly destroyed, which in turn would mean that the inhibitor(s) must be continually produced

in order to prevent the growth of the particular tissue. The arguments in support of such an hypothesis are speculative at present, and alternative ideas can be expressed. However, in favor of the hypothesis mentioned above, the experiments of Medawar (19) and Billingham *et al.* (20) should be quoted, which demonstrate a continuous passage of specific substances from tissues into the circulatory system.

The presence of the mitotic inhibitor in the serum and liver of adult animals and the absence or dilution in serum and liver of rats after a partial hepatectomy throws some light on the mechanism of mitotic regulation in a mammalian organ. It can be postulated that every cell has the potential ability to divide and is prevented from division by an organ-specific inhibitor. The amount of this inhibitor in the serum will thus be the factor responsible for regulating mitosis. A high concentration will inhibit mitosis, and a low one will lead to proliferation. The amount of the inhibitor present in the serum will depend on the amount of tissue by which it is produced, and by the degree of breakdown through catabolic processes. In a normal adult animal both processes seem to be in a state of equilibrium. By alteration of one or both parts of the system, liver or serum, the balance is upset resulting in the onset of mitosis. This can be observed if the liver tissue is partially removed, or the serum diluted (18). The results presented above give experimental support to the hypothesis of Weiss (21), in which the presence of templates and antitemplates is assumed and growth regulation is believed to be based on a negative "feedback" mechanism.

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THE AMINO ACID REQUIREMENTS OF A PERMANENT STRAIN OF ALTERED UTERINE FIBROBLASTS (U12-705)[1]

H. E. Swim and R. F. Parker

Abstract

A permanent line of altered human fibroblasts, strain U12-705, was found to require arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine, and valine for growth in a defined medium supplemented with 2.5% (v/v) dialyzed chick embryo extract and 5% dialyzed horse serum. In the absence of any of the essential amino acids the cells not only fail to proliferate but undergo degenerative changes which increased with time. The omission of alanine, aspartic acid, glutamic acid, glycine, hydroxyproline, and proline either separately or collectively does not alter the rate of growth or result in changes in the appearance of the cells. Cysteine and glutathione are equally as effective as cystine in promoting the growth of U12-705. None of the D-enantiomorphs of the essential amino acids will effectively replace the corresponding L-isomer. Single D-amino acids are not inhibitory when added to the medium in 5 times the concentration of the L-amino acid. The minimum concentrations of essential amino acids which permit optimal proliferation under the conditions employed range from 0.005 to 0.5 m*M*. Essential amino acids with the exception of glutamine, isoleucine, leucine, threonine, and valine are toxic for U12-705 when employed at a concentration of 5 m*M*. Toxic manifestations vary with the amino acid and range from cytologic changes in the cells without a significant decrease in the growth rate to complete inhibition of growth and extensive cellular degeneration.

Introduction

A permanent strain of human fibroblasts designated U12-705, which has been propagated in this laboratory for more than four years, was derived from normal myometrium and underwent physiological and morphological alterations during the first 6 months in vitro (15). As a prerequisite to the investigation of differences in the nutrition of U12-705 and unaltered fibroblasts from the same source, it was considered expedient to examine the requirements of U12-705 both qualitatively and quantitatively. Studies on the nutrition of this strain have been conducted along lines described earlier by Fischer (5, 7), who employed the expedient of supplementing a defined medium with dialyzed serum and plasma and dialyzed embryo extract in an attempt to identify the essential low molecular weight components. This report deals with the amino acids required by U12-705 for proliferation in defined medium supplemented with small quantities of dialyzed chick embryo extract and dialyzed horse serum and with the concentration of each which permits optimal proliferation under these conditions.

Materials and Methods

Medium and Reagents

Medium 705 is composed of the chemically defined solution 703 (10), which contains inorganic salts, glucose, amino acids, vitamins, and coenzymes,

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supplemented with 5% (v/v) chick embryo extract (CEE, 17) and 20% normal horse serum (NHS). Dialyzed horse serum (DHS) and dialyzed CEE (DCEE) were prepared as described by Haff and Swim (8).

Determination of Growth Response to Experimental Media

Stock cultures of strain U12-705 are propagated serially in medium 705 according to methods described previously (15). Duplicate cultures are prepared from 5- to 7-day-old stock cultures as follows. The cells are scraped from the bottom of the T-flasks with a rubber policeman fitted to a Pyrex glass rod and suspended uniformly by vigorous and repeated pipetting. The resulting suspension is diluted with an equal volume of medium 705 and passed through a sintered-glass filter (Ace Glass, Inc., porosity B) to remove clumps of cells. The filtrate is diluted with medium 705 so as to contain 7.5×10^4 cells per ml., as determined by direct counting in a hemocytometer, and 4 ml. aliquots are dispensed into S-20* flasks which are incubated overnight at 37° C. The medium is then removed from groups of three to four duplicate flasks and the cells which adhere to the glass are washed twice with 2-ml. portions of experimental medium. Four milliliters of experimental medium are then added to each flask and these are incubated at 37° C. The medium is replaced on the 3rd day and the experiment is terminated on the 6th day. The cells in each flask are enumerated by a modification of the nuclear counting procedure of Sanford *et al.* (14). The medium in each flask is replaced by 2 to 4 ml. of an aqueous solution containing 6% citric acid and 0.01% crystal violet. A uniform suspension of nuclei, free from cytoplasm, is obtained by shaking the flasks for 5 minutes at a rate of 120 cycles per minute on a reciprocating shaker (Eberbach and Son Co., variable speed blood pipette shaker, equipped with a holder for S-20 flasks). Aliquots of the suspension are then placed immediately into a hemocytometer chamber and the nuclei are counted. The number of cells present at the beginning of the experiment is determined by applying the same procedure to a separate group of duplicate flasks from the same set.

Results

Amino Acids Essential for Proliferation of U12-705

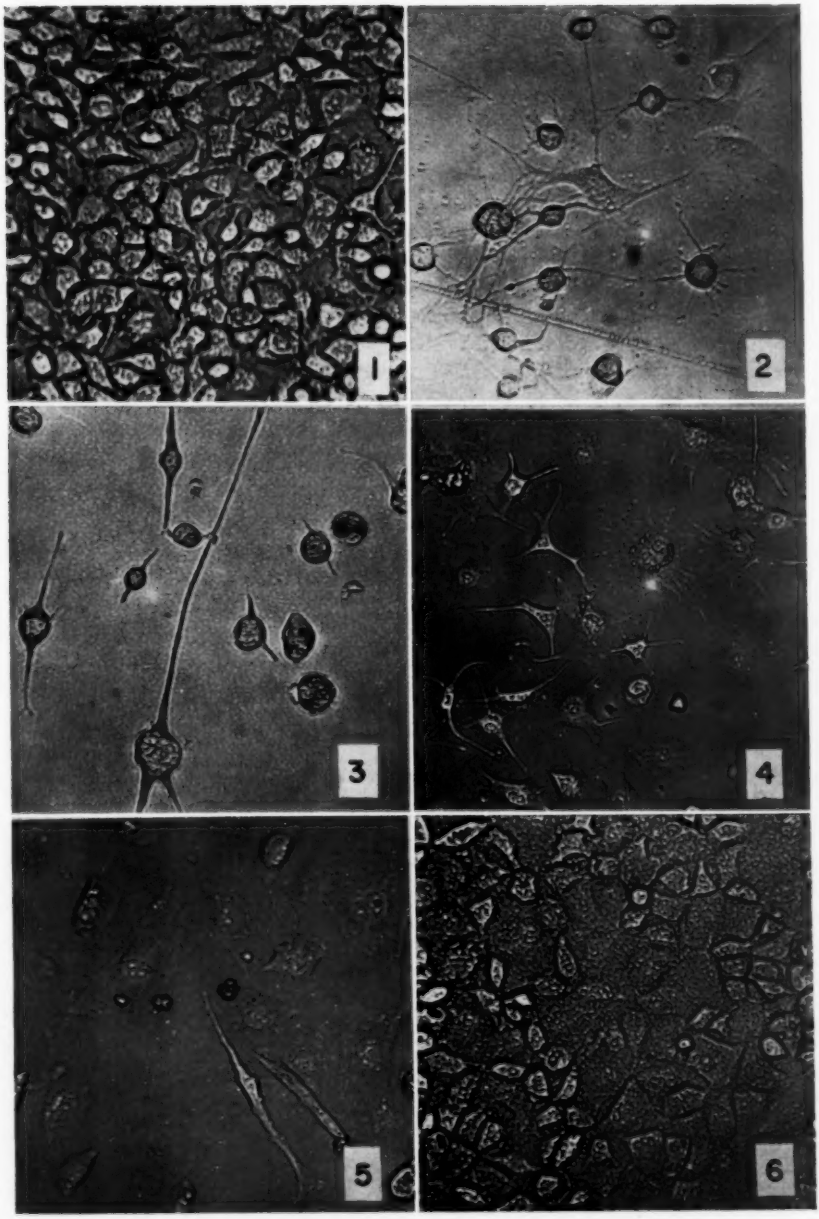
U12-705 cells proliferate at the rate of five- to six-fold in 6 days when subcultured into the basal medium described in Table I. To ascertain which of the amino acids in this medium are essential under these conditions, the

* Flasks with a surface area of 20 cm.^{2} prepared from square Pyrex tubing (2 cm. I.D.). S-20 flasks can be purchased from Euclid Glass Engineering Laboratory, 11310 Wade Park Avenue, Cleveland, Ohio.

FIGS. 1 to 6. Living uterine fibroblasts, strain U12-705, in T-flasks after 6 days in different media. Magnification in all figures is 233 ×.

FIG. 1. A typical culture of U12-705 after 6 days in basal medium described in Table I.
FIG. 2. U12-705 cells after 6 days in the absence of arginine.
FIG. 3. U12-705 cells after 6 days in the absence of valine.
FIG. 4. U12-705 cells after 6 days in the absence of methionine.
FIG. 5. U12-705 after 6 days in a medium containing 5 m*M*. tryptophan.
FIG. 6. U12-705 cells after 6 days in a medium containing 5 m*M*. arginine.

PLATE I



Swim and Parker—Can. J. Biochem. Physiol.

growth response of U12-705 is determined quantitatively in experimental media lacking in single amino acids. The results are summarized in Table II. When arginine, cystine, cysteine, glutamine (considered as an amino acid for purposes of this discussion), glutathione, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, or valine are omitted separately from the medium, the cells not only fail to proliferate but degenerate as is indicated by the fact that, with the exception of media deficient in either glutamine or methionine, the number of cells recovered after 6 days is less than the inoculum. Some examples of the cytologic changes which are usually evident after 2 to 3 days and increase with time are shown in Figs. 2, 3, and 4. In spite of the fact that there is no net decrease in the number of cells after 6 days in the absence of glutamine or methionine, cytologic alterations which are characteristic of cellular degeneration are evident after 3 to 4 days (see Fig. 4), and by 10 days the number of cells is reduced to about 0.5 that added in the inoculum. The omission of alanine, aspartic acid, glutamic acid, glycine, hydroxyproline, proline, and serine either separately or collectively does not alter the growth response or result in changes in the appearance of the cells.

TABLE I

COMPOSITION OF BASAL MEDIUM[a] USED TO DETERMINE AMINO ACID REQUIREMENTS OF U12-705

Compound[b]	Mg. per liter	Compound	Mg. per liter
Alanine	25	Biotin	0.01
Arginine	70	Calciferol	0.10
Aspartic acid	30	Choline	0.50
Cysteine	200	Folic acid	0.01
Cystine	20	Inositol	0.05
Glutamic acid	75	Menadione	0.01
Glutamine	100	Nicotinamide	0.025
Glutathione	10	Calcium pantothenate	0.01
Glycine	50	Pyridoxal	0.025
Histidine	20	Riboflavin	0.01
Hydroxyproline	10	Thiamin	0.01
Isoleucine	20	α-Tocopherol phosphate	0.01
Leucine	60	Vitamin A	0.10
Lysine	70	Diphosphopyridine nucleotide (95%)	0.8
Methionine	15	Triphosphopyridine nucleotide (95%)	0.4
Phenylalanine	25	Coenzyme A (75%)	0.3
Proline	40	Ethyl alcohol	16.0
Serine	25	Ferric nitrate	0.1
Threonine	30	Sodium penicillin G	60.0
Tryptophan	10	Streptomycin sulphate	25.0
Tyrosine	40		
Valine	25		
Ascorbic acid	50.0		

[a]Medium also contains ingredients of Earle's balanced salt solution (3) and is supplemented with 2.5% DCEE and 5% DHS.
[b]All amino acids of the L-configuration.

TABLE II

EFFECT OF OMITTING COMPOUNDS SEPARATELY FROM BASAL MEDIUM ON PROLIFERATION OF STRAIN U12-705

Compound omitted	Inoculum × 10^4	Growth response[a]
None	30	5.5
Arginine	33	0.5
Cystine[b]	29	0.5
Cysteine[c]	24	0.5
Glutamine	36	1.1
Glutathione[d]	28	0.8
Histidine	32	0.6
Isoleucine	38	0.7
Leucine	35	0.6
Lysine	45	0.5
Methionine	36	1.0
Phenylalanine	31	0.7
Threonine	38	0.5
Tryptophan	25	0.5
Tyrosine	25	0.3
Valine	25	0.6
Alanine	27	5.1
Aspartic acid	27	4.7
Glutamic acid	27	5.3
Glycine	32	5.3
Hydroxyproline	30	5.6
Proline	30	4.8
Serine	27	5.0

[a]Fold increase in cell number for a period of 6 days. Average values for three to five experiments.
[b]Cysteine and glutathione were also omitted from the basal medium.
[c]Cystine and glutathione were also omitted.
[d]Cystine and cysteine were also omitted.

Growth Response as a Function of Amino Acid Concentration

The growth rate of U12-705 as a function of the concentration of each of the essential amino acids is illustrated in Table III. With increasing concentrations of a single amino acid there is a corresponding increase in the number of cells until a maximal response is obtained at concentrations which range from 0.005 to 0.5 m*M*. Moderate increases in the concentration of amino acids above that which permits maximal proliferation are without effect, but a number of amino acids are toxic at a concentration of 5 m*M*. Toxic manifestations are recognized either by inhibition of growth or by cytologic changes in the cells. The latter may (Fig. 5) or may not (Fig. 6) be coincident with a decrease in rate of growth (Table III).

Failure of D-*Amino Acids to Replace Corresponding* L-*Enantiomorphs*

The results presented in Table IV indicate that the D-enantiomorphs of the essential amino acids will not substitute for the L-isomers. Single D-amino

TABLE III

GROWTH RESPONSE OF STRAIN U12-705 AS A FUNCTION OF CONCENTRATION OF INDIVIDUAL AMINO ACIDS

Amino acid	Inoculum × 10^4	Amino acid concentration, mM[a]: 0	0.002	0.005	0.01	0.02	0.03	0.05	0.1	0.2	0.5	5.0	Degree of toxicity at 5 mM[c]	Minimal concn. permitting growth[d]	Concn. permitting optimal growth
		Fold increase in cell number after 6 days[b]													
Arginine	33	0.6			0.8			1.8	3.7	6.5	5.9	5.1	+	0.05	0.2
Cystine	35	0.8		1.9	4.5	5.1	5.3	5.4			4.6	4.0	+++	0.01	0.02
Cysteine	36	0.8			1.4	2.1	4.1	5.6	5.5		5.1	3.5	++	0.02	0.05
Glutamine	30	1.2[e]						1.7	2.7	4.2	5.6	6.0	++	0.1	0.5
Glutathione	35	0.8			1.2	1.9	3.8	5.7	5.4		5.9	4.4	+	0.02	0.05
Histidine	31	0.6	1.5	3.2	5.6			5.4	5.0		5.2	2.0	+++	0.003	0.01
Isoleucine	35	0.7			1.6	2.9	4.2	5.7	6.1		5.5	5.4	0	0.02	0.05
Leucine	31	1.4[e]			1.5	2.6	4.6	5.5	5.0		6.4	5.9	0	0.02	0.05
Lysine	37	0.9			0.9	2.1	3.7	5.8	5.3		5.1	5.6	+	0.02	0.05
Methionine	35	1.2[e]		1.2	2.5	4.3	5.8	5.7	5.9		5.8	3.1	+++	0.01	0.03
Phenylalanine	40	0.8		1.9	4.2	5.9	5.2	6.1			6.0	4.2	++	0.005	0.02
Threonine	31	0.9				1.2	1.6	3.8	5.9	5.4	5.7	5.8	0	0.05	0.1
Tryptophan	26	0.5	2.4	5.7	5.6	5.6		5.9			5.2	1.0	+++	0.002	0.005
Tyrosine	27	0.3		1.2	2.4	4.6	5.8	5.6	5.2		5.3	0.6	++++	0.01	0.03
Valine	28	0.6				1.7	2.8	4.2	6.5		6.2	6.1	0	0.03	0.10

[a] A number of amino acids tested at concentrations of 0.003, 0.075, 0.15, and 1 mM are not shown in Table III.
[b] Average values for three to five experiments.
[c] Extent of cytologic changes observed in the cells after 6 days. These range from the appearance of enlarged, epithelial-like cells (+) to severe damage and cell destruction (++++).
[d] Minimal concentration which permits growth in the absence of cytologic changes.
[e] Although number of cells remained constant or increased slightly, cellular degeneration was evident after 4 to 6 days.

TABLE IV

LACK OF GROWTH RESPONSE OF STRAIN U12-705 TO D-AMINO ACIDS

Amino acid[a]	L-Amino acid for maximal growth, m*M*	Fold increase in cell number after 6 days: Concentration of D- or L-amino acid[b] 0	1 × D	2 × D	5 × D + L	1 × L
Cystine	0.02	0.8	0.6	0.9	6.3	6.8
Histidine	0.01	1.0	0.9	0.8	6.0	6.2
Isoleucine	0.05	0.9	0.7	0.8	6.3	6.4
Leucine	0.05	1.0	1.8	0.6	7.0	7.1
Methionine	0.03	1.2	1.9	1.0	5.9	6.2
Phenylalanine	0.02	0.5	0.7	0.6	6.1	5.9
Threonine	0.1	0.5	0.5	0.4	6.1	5.6
Tryptophan	0.005	0.3	0.4	0.4	5.9	5.9
Tyrosine	0.03	0.8	0.7	0.6	6.4	7.1
Valine	0.10	0.3	0.3	0.5	5.9	6.4

[a]In these experiments the amino acids of basal medium shown in Table I were substituted by the essential amino acids at 4 times the concentration required for optimal proliferation (Table III).
[b]Expressed as multiples of concentration of amino acid which permits maximal proliferation as shown in column 2.

acids also do not inhibit growth significantly when added in a concentration exceeding that of the L-enantiomorph by a factor of 5. The D-isomers of arginine and lysine are also inactive as judged by the growth response of the cells to graded concentrations of the corresponding racemic mixtures.

Discussion

The amino acids required by U12-705 for proliferation in a defined medium supplemented with 2.5% DCEE and 5% DHS are the same as those required by mouse fibroblasts, strain L (1), and human epidermoid carcinoma cells, strain HeLa (2). There are, however, a number of exceptions to this common pattern. Rabbit fibroblasts, strain RM3-56, require serine in addition to those amino acids found essential for U12-705 (8) and the Walker rat carcinoma has been shown to require asparagine (13). On the other hand, Morgan and Morton (12) reported that glutamine and isoleucine are not required for the survival of depleted chick embryo fibroblasts. This raises the question of whether the amino acids which are necessary for limited periods of survival in vitro may be different from those required for growth in view of the fact that glutamine has long been considered an essential amino acid for the proliferation of cells from explanted chick embryo tissue (4, 6). Similarly, the requirement of cystine for survival of chick embryo fibroblasts is relatively specific (12) whereas glutathione and cysteine are as effective as cystine for the growth of U12-705 and RM3-56.* Cysteine also completely replaces cystine in the case of strains L (1) and HeLa (2). In this connection, it is to be noted that additional amino acids may be required by U12-705 for

* Unpublished experiments.

growth or for optimal proliferation under conditions of continuous serial propagation. This point could not be established in the present studies since U12-705 proliferates for only one to two passages in the basal medium employed.

The concentrations of the essential amino acids which permit optimal proliferation of U12-705 vary over a range of 0.005 to 0.5 m*M*. In this respect U12-705 is very similar to strains L (1), HeLa (2), and RM3-56 (8) which have been studied under analogous experimental conditions. It is to be noted, however, that each strain of cells possesses a certain degree of individuality not only with regard to the concentration of individual amino acids required for optimal growth, but also with respect to their response to either deficiencies or high concentrations of amino acids. For example, U12-705 is unique among the strains studied to date in that a relatively high concentration of arginine (0.2 m*M*) is necessary for optimal proliferation. Toxic manifestations elicited by amino acids when employed in concentrations of 5 m*M* or greater are a function of both the amino acid and the strain of cells. These observations point up the importance of employing balanced media, which are formulated on the basis of experiments, with the cells being studied. This conclusion is exemplified by the data presented in Table IV which demonstrate that the rate of growth of U12-705 is improved significantly when the relative concentrations of the essential amino acids in the medium are adjusted to meet the requirements of this strain of cells (compare Tables II, III, and IV).

Strains L, HeLa, and U12-705 degenerate in a few days when deprived of any of the essential amino acids. In the case of RM3-56, cellular degeneration is observed during the first week only when glutamine or cystine is omitted from the medium. It is of particular interest that the adverse effects of unbalanced metabolism resulting from amino acid deficiencies appear to be more pronounced with morphologically altered (L and U12-705) or malignant cells (L and HeLa) than with newly established strains* or permanent lines with unaltered morphological characteristics (RM3-56). The extent to which this may be generally applicable and the degree to which differences in the response to amino acid deficiences reflect variations in the over-all physiology of various strains remain to be determined.

The demonstration that cystine, glutamine, and tyrosine are required by fibroblasts cultured in vitro whereas they are not required for growth or for the maintenance of nitrogen balance in the corresponding species (11) cannot be considered as indicative of the degree to which nutritional synergism exists between cells with different physiological functions in the intact animal. Evidence was presented in an earlier report that U12-705 is an example of a strain of fibroblasts derived from normal tissue which have undergone nutritional alterations in vitro (15). It has also been demonstrated that the nutrition of a particular strain of cultured cells is within limits a function of the composition of the medium in which it is serially propagated (9, 16, 18).

* Unpublished experiments.

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ISOLATION OF A STEROID-LIKE SUBSTANCE FROM URINE AND CITROUS FRUITS[1]

WOJCIECH NOWACZYNSKI, THOMAS SANDOR, ERICH KOIW, R. NORMAN JONES, AND JACQUES GENEST

Abstract

The isolation and the partial chemical and physicochemical characterization of a chloroform-soluble substance obtained from human urine and citrous fruit juices are presented. The urinary compound, designated as compound III, is present mostly as a conjugate hydrolyzed by animal β-glucuronidase whereas it is in free form in fruit juices. It is present in the urine of normal males and females and in a number of pathological conditions. Its urinary excretion is significantly increased as a result of diets rich in potassium (250 meq./day). In various paper chromatographic systems, the compound has a mobility similar to that of aldosterone. Detailed study of its chemical and physicochemical characteristics, including oxidative degradation and infrared and elementary analysis, suggests a steroidal or sesquiterpinoidal structure. However, at the present time, the data obtained do not permit the final elucidation of the chemical formula of compound III.

Introduction

Previous work from this laboratory (22, 24, 25) has shown the presence of seven different ultraviolet-absorbing substances in the aldosterone fraction obtained after the successive chromatographic purification (propylene glycol/toluene; toluene – ethyl acetate 90:10/55% aqueous methanol systems) of the crude neutral extract of urine (12). The present report describes the chemical, physicochemical, and certain biological properties of a compound frequently found in this fraction and referred to as compound III. In March 1957, Dr. Gray from King's College Hospital in London brought our attention to the similarity of our compound III to a substance isolated by his group (13, 14) from crude urinary extracts and citrous fruit juices. This compound, detected in the first paper chromatographic separation of crude urinary extracts and designated as X_6, was found by us to be identical with our compound III isolated in this laboratory from the aldosterone fraction of urinary extracts and from fruit juices.

Method

Solvents and Reagents

1. Chloroform: Mallinckrodt, Analytical Reagent. Redistilled and preserved with ethanol (1%).

2. Methanol: Merck Reagent. Redistilled.

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Contribution from the Clinical Research Department, Hotel-Dieu Hospital, Montreal, Que. and the Division of Pure Chemistry, National Research Council, Ottawa. With the technical assistance of Robert Tellier, Isabelle Morin, Alice Laflamme, Pauline Robinson, and Roland Lauzon. Reported at the Annual Meeting of the Endocrine Society held in New York City, June 1957. This work was generously supported through grants of the Ministries of Health (Federal–Provincial Plan); the Life Insurance Medical Research Fund, New York; the Upjohn Company, Kalamazoo; and the Ciba Company, Montreal.

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3. Ethanol: Gooderham & Worts. Twice distilled, the second time from dianisole-bis-4,4-(3,5-diphenyl) tetrazolium chloride (blue tetrazolium, Dajac) and NaOH pellets.

4. Benzene: Mallinckrodt, Analytical Reagent. Redistilled and purified according to Schindler *et al.* (32).

5. Ethyl acetate: Mallinckrodt, Analytical Reagent. Redistilled.

6. Tertiary butyl alcohol: Fisher, Reagent Grade.

7. Acetone: Mallinckrodt, Analytical Reagent. Redistilled.

8. Ethylene glycol: Fisher, Reagent Grade.

9. Isooctane: Eastman, Practical Grade.

10. Toluene: Merck, Reagent Grade.

11. Propylene glycol: Eastman.

12. Concentrated sulphuric acid: Merck, Reagent Grade.

13. Phosphoric acid, 85%: Baker's Analyzed.

14. Ligroine: Eastman, Practical, D 0.67–0.69. Purified according to Hegedüs *et al.* (15).

15. Hyflo Super Cell: John's Manville. Purified according to Hegedüs *et al.* (15).

16. Phosphorus pentoxide: Baker's Analyzed.

17. Hydrochloric acid: Mallinckrodt, Analytical Reagent.

18. Sodium bismuthate: Fisher Certified Reagent.

19. Acetic acid glacial: Baker's Analyzed.

20. Chromium trioxide: Mallinckrodt, Analytical Reagent.

21. Periodic acid: C. P. Smith Chemical Co.

22. Cyclohexane: Fisher, Reagent.

23. Whatman No. 2 paper "especially selected for chromatography" was used for all the paper chromatographic separations. This paper is washed prior to chromatography in a Soxhlet apparatus for 72 hours with a 1:1 v/v mixture of benzene and methanol.

Method of Isolation of Compound III from Urine and Citrous Juices

A. Hydrolysis and Extractions

Urines are adjusted to pH 1 and extracted immediately four successive times with 0.2 volume of chloroform and are re-extracted after incubation with animal β-glucuronidase (300 units/ml. of urine) at pH 4.5 for 24 hours at 37° C. Fresh citrous juices are extracted immediately with chloroform without pH adjustment or incubation.

After extraction, the pooled chloroform extracts are washed twice with 0.1 volume of 0.1 *N* sodium hydroxide and twice with distilled water. The washings are back-extracted with equal volumes of chloroform. After evaporation of the extract in a rotary evaporator, the residue is submitted to the following successive chromatographic procedures. (For technical details, refer to our previous publication (22).)

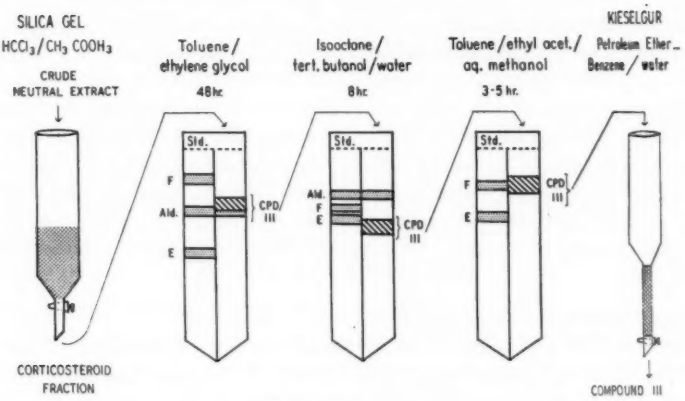

FIG. 1. See text.

B. Purification Procedures (Fig. 1)

(*a*) *Silica gel column fractionation of the crude neutral extract* (20, 25).—This is performed on a 2.5 g. silica gel column (7 mm. in diameter). Elution is done with two successive mixtures of chloroform–acetone 99:1 v/v (50 ml.) and of chloroform–acetone 1:1 v/v (100 ml.). Compound III is eluted in the corticosteroid fraction with the $CHCl_3$–acetone 1:1 v/v mixture.

(*b*) *Paper chromatographic separation in ethylene glycol/toluene system* (*23*).—The corticosteroid fraction from the silica gel column fractionation is run in this system for 48 hours at room temperature. Compound III is less polar than hydrocortisone and more polar than aldosterone.

(*c*) *Paper chromatographic separation in the system: tertiary butanol* (*250 ml.*) – *water* (*450 ml.*)/*isooctane* (*500 ml.*) (*11*).—In this system, compound III is well separated from aldosterone, and is slightly less polar than cortisone.

(*d*) *Paper chromatographic separation in the system: 55% aqueous methanol/toluene – ethyl acetate 90:10* (*v/v*) (*6*).—Compound III, which is separated from most contaminants by the previous chromatographic systems, has a R_f value of 0.27 in this system. Compound III is still, at this point, contaminated in varying proportions with blue tetrazolium reducing substances. It is localized in all systems with the aid of an ultraviolet scanning lamp with a maximal emission of 254 mμ.* The paper zones containing the compound III are eluted, using a modified Haines elution tube with either 95% ethanol or 80% aqueous methanol (22).

(*e*) *Kieselgur column chromatography* (*32*).—Solvents and the Kieselgur are purified according to Hegedüs *et al.* and Schindler *et al.* (15, 32). Stationary phase: 5 g. of Hyflo Super Cell (Johns Manville) mixed with 5 g. of water (chromatographic column: 7 mm. internal diameter). Successive eluents: (1) 200 ml. petroleum ether; (2) 150 ml. petroleum ether+50 ml. benzene; (3) 100 ml. petroleum ether+100 ml. benzene; (4) 200 ml. benzene. The

*Mineralite, Model RV 71, Research Equipment Corporation, California.

purpose of this final column chromatography is to liberate compound III from blue tetrazolium reducing substances which cannot otherwise be separated from it. At the same time, compound III is freed from other materials eluted from the chromatographic paper. Compound III is eluted in fraction No. 3.

(f) High vacuum distillation.—The crystallization of the free or acetylated compound III has not yet been satisfactorily achieved, but the material was further purified by high vacuum distillation. The distillation was performed by using a microdistillation apparatus in a vacuum of 0.003 mm. of Hg. Distillation temperatures: free compound; 110–115° C.; acetylated compound, 155–160° C.

C. Quantitation of Compound III

Compound III was quantitatively measured by its ultraviolet absorption at 239 mμ in 95% ethanol using a Beckman DU quartz spectrophotometer. The following formula was used:

$$\text{microgram per liter} = (Mw \times \text{O.D.}_{239} \times 1000)/\epsilon$$

where Mw = molecular weight (346, hypothetical),

ϵ = molecular extinction coefficient (15800, hypothetical).

A hypothetical molecular weight was used until sufficient material became available for a molecular weight determination.

Alternative Procedures for Isolation

(*a*) The continuous extraction of urine adjusted at pH 1 for 30 hours in a Cohen type extractor was also used as hydrolysis extraction procedure. Only a small amount of compound III could be extracted from urine by this method as compared to that obtained following hydrolysis with the animal β-glucuronidase preparation.

(*b*) The propylene glycol – toluene chromatographic system (37) was also used as the first paper chromatographic separation of the corticosteroid fraction. In this system compound III migrates with the cortisone–aldosterone zone.

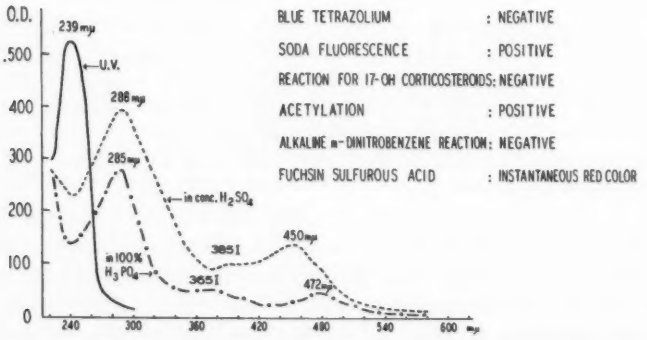

FIG. 2. Description of some physicochemical properties of compound III, including absorption spectra of the free compound in 95% ethanol, in concentrated sulphuric acid, and in "100%" phosphoric acid.

TABLE I

PAPER CHROMATOGRAPHIC MOBILITY OF COMPOUND III

Toluene/propylene glycol system	Aldosterone–cortisone zone
Toluene/ethylene glycol system	$R_{cortisone}$, 0.56
Benzene/55% aqueous methanol system	R_f, 0.34
Toluene – ethyl acetate 90:10 (v/v)/55% aqueous methanol system	R_f, 0.27
Tertiary butanol/isooctane–water system	$R_{cortisone}$, 1.14

Results and Discussion

Characterization of Compound III (Fig. 2, Table I)

The following data refer to the compound extracted from urines as well as from citrous fruit juices. The material from both sources shows identical chemical and physicochemical properties (Figs. 1–5).

1. The substance dissolved in 95% ethanol absorbs ultraviolet light with a maximum at 239 mμ.

2. Absorption spectrum in concentrated sulphuric acid ($E^{1\%}_{1cm.}$) (3, 4):

Maxima, mμ	Minima, mμ
285 (251)	240 (105)
345I (66)	
450 (70)	370 (46)

3. Fluorescence with 10% sodium hydroxide: positive.

4. Absorption spectrum in 100% phosphoric acid ($E^{1\%}_{1cm.}$) (26, 27):

Maxima, mμ	Minima, mμ
285 (314)	
365 (44)	240 (97)
472 (31)	430 (16)

5. Reduction of blue tetrazolium: negative (21).

6. Alkaline metadinitrobenzene reaction: negative.

7. S.P.R. reaction (sulphuric–phosphoric acids reagent) for presence of Δ^4-pregnene-17α,21-diol-3,20-dione structure: negative (35).

8. Schiff's reagent (fuchsin sulphurous acid): instantaneous red color.

9. Acetylation: the compound is acetylated with acetic anhydride in pyridine at room temperature for 48 hours (33).

R_f value of the acetate in the toluene – ethyl acetate/aqueous methanol system is 0.82 in contrast to 0.27 for the free compound.

10. Molecular weight determination (Rast's camphor method) (28, 29): 317. The molecular extinction coefficient calculated with this molecular weight for 239 mμ is 16,000.

11. Elementary analysis:

(*a*) A sample of 3.873 mg. of compound III acetate purified by high vacuum distillation was sent to Dr. Peter S. Cammarata and Dr. R. Dillon of the Searle Company, Chicago, who found a carbon content of 66.6%, hydrogen 8.7%, and oxygen by difference 24.7%.

(*b*) Another sample of compound III acetate purified only by the chromatographic procedure described above was kindly analyzed by Dr. Lyman

Craig of the Rockefeller Institute for Medical Research, New York. The analysis gave the following findings: carbon 68.82%, hydrogen 8.88%, and oxygen by difference 22.3%.

12. Oxidative degradation studies:

Preparative oxidations were performed by using periodic acid (34), sodium bismuthate (5), and chromium trioxide in 90% acetic acid (36). Compound III readily reacted with all three agents and yielded products different from the starting material and insoluble in saturated sodium bicarbonate. The paper chromatographic mobilities of the oxidation products showed a drastic change towards decreasing polarities as compared to that of compound III. These products were chromatographed on activated alumina (Activated Alumina, F-20, the Aluminum Co. of America) and eluted with a mixture of benzene, 0.1% ethanol (1). Ultraviolet absorption spectra in ethanol, and the spectra in concentrated sulphuric and "100%" phosphoric acid as well as chromatographic mobilities are indicated in Table II and Fig. 3.

TABLE II

PHYSICOCHEMICAL PROPERTIES OF THE OXIDATION PRODUCTS

Oxid. agent	Recovery, %	U.V. max., mμ	Conc. H_2SO_4 chromogen		100% phos. chromogen		17 K,* reaction	Mobilities	
			Max.	Min.	Max.	Min.		Bush C, R_f	17 K, system,† R_t‡
KIO_4	97	238	280	245	280	238	Violet	0.87	1.99
NaBiO	86.7	238	283	242	277	250	Violet	0.87	1.99
CrO_3	89.7	238	283	240	280	236	Violet	0.87	1.99

*Alkaline *m*-dinitrobenzene reaction (7).
†Cyclohexane–benzene 1:1 saturated with propylene glycol (18)
‡Mobilities relative to testosterone = 1.

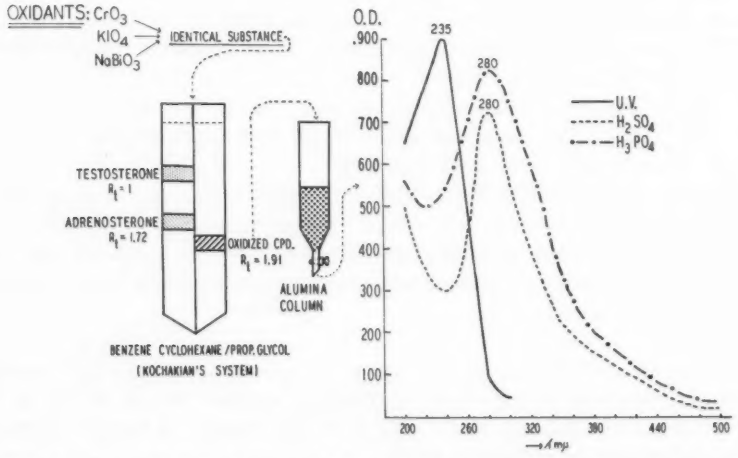

FIG. 3. The oxidation of compound III isolated from urine or citrous juices, with CrO_3, $NaBiO_3$, or KIO_4, yields products with identical chromatograph c mobilities and physicochemical characteristics.

TABLE III

SPECTROPHOTOMETRIC DATA OF ADRENOSTERONE

	Maximum, mμ	Minimum, mμ
U.V. absorption (in 95% ethanol)	235	– (8)
Conc. sulphuric acid chromogen	283	230 (3)
"100%" phosphoric acid chromogen	280	238 (26, 27)

From these data the following conclusions could be drawn:

(1) The three products are identical. This would indicate, if the compound has a steroidal structure, the absence of a OH group on the skeleton.

(2) The spectra are similar in most respects to those given by Δ^4-androstene-3,11,17-trione (8, 3, 26) (Table III). The oxidation products readily react with the alkaline metadinitrobenzene reagent (7) giving a violet color with a peak at 500–505 mμ. In a paper chromatographic system of cyclohexane – benzene – propylene glycol, these products showed a slightly greater mobility ($R_{\text{testosterone}}$ 1.99) than Δ^4-androstene-3,11,17-trione ($R_{\text{testosterone}}$ 1.72). The ultraviolet-absorbing properties were not affected by either oxidizing agent.

Spot test reactions described by Axelrod (2) were also performed on compound III. Amounts of 15–30 μg. were deposited on Whatman No. 2 filter paper strips in an area not exceeding 1 sq. cm. Oxidation with potassium periodate gave a product reacting with a violet color when sprayed with alkaline metadinitrobenzene. On the other hand, CrO_3 in acetic anhydride failed to yield a triphenyl tetrazolium reducing material.

13. Infrared spectrum and structural analysis:

In Fig. 4 the infrared spectrum of a concentrate of compound III is compared with the spectrum of the product obtained from grapefruit juice; the spectra of the corresponding acetates are compared in Fig. 5. The curve shown in Fig. 6 was obtained for the acetylated urinary compound after further fractionation. This material, though still not crystalline, is the purest yet obtained and gave the carbon and hydrogen analyses reported above in section 11 (*b*). The spectrum of the product obtained by chromic acid oxidation of compound III is shown in Fig. 7. All spectra were determined on a Perkin–Elmer Model 21 spectrophotometer using a sodium chloride prism.

The broadness of the bands and lack of structural detail in the spectra shown in Figs. 4 and 5 are indicative of the impure state of the products. Nevertheless the curves of the materials derived from the two sources resemble each other closely and the only significant difference is in the 1750–1700 $cm.^{-1}$ region of Fig. 4 where the product from grapefruit juice shows a fully resolved band and the urinary compound a weaker inflection. The spectra of the acetates show no significant differences, and this band may well arise from a contaminant. When these curves are considered together with the chemical and chromatographic behavior they provide strong evidence for the identity of the two materials.

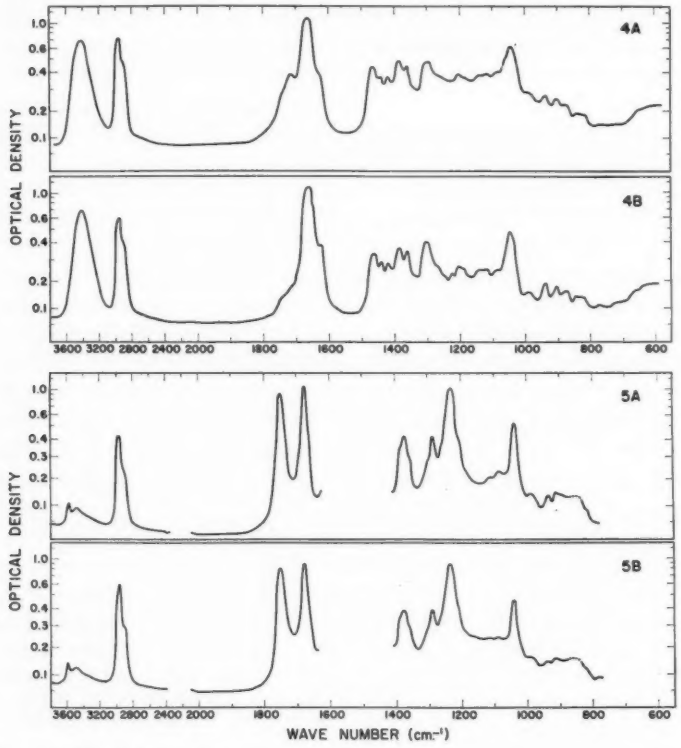

FIG. 4. Infrared spectrum.
Curve A, product from grapefruit juice. Curve B, compound III from urine. (KBr disk spectra.)

FIG. 5. Infrared spectrum.
Curve A, acetylated product from grapefruit juice. Curve B, acetylated compound III from urine. (CS_2 soln. 1 mm. cell.)

It is not possible from the infrared spectrum to state with certainty that the compound is a steroid, but if this is assumed, the strong C—O stretching band (1660 cm.$^{-1}$ in $CHCl_3$ solution) could be assigned to the Δ^4-3-ketone group. The weaker band at 1618 cm.$^{-1}$ in Figs. 4 and 6 can be assigned to the C—C stretching vibration of this group. In Fig. 5 this is obscured by solvent absorption. If the 1712 cm.$^{-1}$ band of Fig. 4A is attributed to impurity, no other carbonylic functional groups are present in the free compound. Strong absorption between 3600 and 3400 cm.$^{-1}$ in Fig. 4 indicates the presence of several hydroxyl groups.

The spectrum of the acetylated product exhibits an additional carbonyl peak at 1745 cm.$^{-1}$ in CS_2 solution and at 1737 cm.$^{-1}$ in $CHCl_3$ solution. This band lies in the high end of the normal range for the "unperturbed" acetate group; it is suggestive of acetylation in the side chain of a C_{21} steroid rather

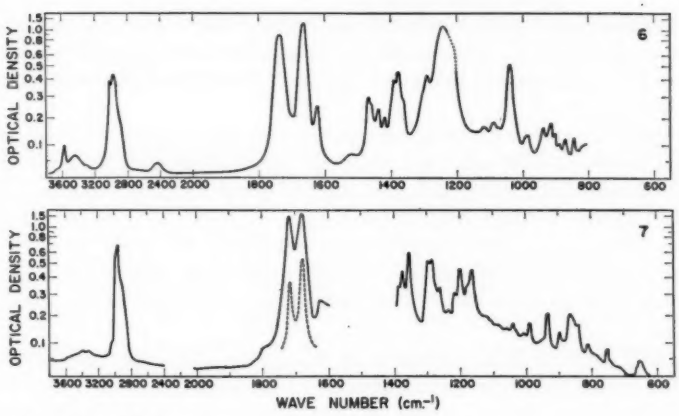

FIG. 6. Infrared spectrum.
Acetylated compound III from urine after high vacuum distillation. ($CHCl_3$ soln. 0.1 mm. cell.)

FIG. 7. Infrared spectrum.
Product obtained by oxidation of compound III from urine with CrO_3. (CS_2 soln. 1 mm. cell.)

than in the ring system. A band at this position has been noted in 20β,21-diacetates (17), but in this case a diacetate structure is highly unlikely as the intensity of the 1737 cm.$^{-1}$ band of Fig. 6 is significantly less than that of the 1659 cm.$^{-1}$ band, whereas it should be stronger in a diacetoxy-Δ^4-3-ketone structure (cf. 20β,21-diacetoxy-17α-hydroxy-Δ^4-pregnen-3-one in Chart 185 of reference 10). The intensity of the acetate C—O stretching band at 1240 cm.$^{-1}$ is also best reconciled with a monoacetate structure. The presence of the typical O—H stretching band at 3580 cm.$^{-1}$ in Fig. 6 establishes the presence of non-acetylated hydroxyl in the acetate. One cannot state positively the number of such hydroxyl groups present, but if a value of 700 is assumed for $\epsilon^{(a)}_{max}$ for the Δ^4-3-ketone peak, a value of 52 is obtained for the intensity of the 3580 cm.$^{-1}$ peak and this is in good accord with intensities observed for monoalcohols under comparable conditions (19); in any event it would seem unlikely that more than two such groups are present.

This analysis of the infrared spectra, therefore, suggests a structure for compound III containing one conjugated ketone group, one readily acetylated hydroxyl group, and one or possibly two additional hydroxyl groups not acetylated by acetic anhydride and pyridine at room temperature. Few spectra of such steroids are available for comparison purposes, but comparisons with the spectra of 17β-acetoxy-11β-hydroxy-Δ^4-androsten-3-one and 17α,20β-dihydroxy-Δ^4-pregnen-3-one in Charts I.172 and II.461 of references 10, 30 showed no close similarities.

Steroid structures of these types in both the C_{19} and C_{21} series would require carbon analyses higher by 2 or 3% than the values obtained for this product, and the analytical data would be in better accord with a C_{21} steroid monoacetate containing five or six oxygen atoms. Such an empirical formula could

best be reconciled with the infrared spectrum if the additional oxygen were present in an ether linkage. Both the acetate and the non-acetylated compound exhibit a prominent band at 1040 cm.$^{-1}$ which could be associated with such a group.

The spectrum of the chromic acid oxidation product (Fig. 7) possesses two carbonyl bands at 1716 and 1673 cm.$^{-1}$ in carbon disulphide solution, and no hydroxyl absorption. The carbonyl band of lower frequency can be assigned to the conjugated ketone group of compound III unchanged by oxidation. If a conventional C_{21}-steroidal structure is assumed, the band at 1712 cm.$^{-1}$ suggests a carbonyl group either at C(20) or in a six-membered ring. The prominent peak at 1354 cm.$^{-1}$ is consistent with a C(21)-methyl group of a 20-ketone.

It is most significant that there is no band near 1745 cm.$^{-1}$ assignable to a 17-ketone group and this would appear to exclude C(17) as a site for the non-acetylatable hydroxyl group of compound III. This hydroxyl group could be at C(11) giving rise by oxidation to an 11-ketone. However the relative intensities of the 1712 and 1673 cm.$^{-1}$ peaks suggest that only one non-conjugated carbonyl group is present and if this is assigned to an 11-ketone it is difficult to visualize a reasonable structure for the side chain. Furthermore this is not consistent with the identity of the product obtained by oxidation with chromium trioxide, periodic acid, and sodium bismuthate (see above). The spectrum in Fig. 7 shows no close similarity to that of Δ^4-pregnene-3,20-dione or Δ^4-pregnene-3,11,20-trione (Charts 119 and 126 of reference 10).

Since the steroidal nature of compound III has not been established, other structures must also be considered. Its occurrence in citrous fruits suggests a possible association with compounds of the limonin type (31, 16). These have been isolated and identified as the bitter principles of this type of fruit, and are based on a C_{26} carbon skeleton. Compound III is not to be identified with limonin itself since the latter contains two lactone groups with characteristic infrared absorption at 1760 cm.$^{-1}$. Compounds related to limonin, however, cannot be excluded on the basis of the available evidence nor can sesquiterpinoid or triterpinoid structures.

14. Biological activity:

Preliminary observations showed a slight to moderate natriuretic effect of compound III in adrenalectomized rats. These findings were not supported by further studies in intact and adrenalectomized dogs in our laboratory nor by studies from other groups in adrenalectomized rats.*

15. Compound III, both from urinary and citrous fruit sources, was also isolated according to the above described extraction procedure at Merck, Sharp & Dohme Laboratories by Drs. N. Brink and E. A. Ham.† They confirmed our findings regarding the compound's mobility on paper in different chromatographic systems, its ultraviolet spectrum in ethanol, and the

*We wish to express our thanks to Dr. H. C. Stoerk, Merck & Co., and to Dr. C. M. Kagawa, Searle & Co., for their collaboration.

†Personal communication.

spectrum in concentrated sulphuric acid. Acetylation and oxidation with periodic acid and sodium bismuthate yield products identical with those obtained in this laboratory.

Occurrence

This compound is found in urines obtained from normal men and women, in pregnancy, in essential and malignant hypertension, and in Cushing's syndrome. No effort was made to study other conditions. Its urinary excretion varies from a few micrograms to several milligrams per day. A high intake of potassium (240–300 meq./day) markedly increases the urinary excretion of compound III. This effect of potassium was confirmed on several occasions (25). A 30- to 100-fold increase was observed during the high K intake period as compared to the control values. Once the potassium administration was stopped, the urinary compound III excretion dropped to the control level.

These experiments were confirmed at the laboratories of Merck, Sharp & Dohme, where 0.8 mg. of compound III per liter was found in a pool of urine of potassium-loaded individuals. The amounts of compound III isolated from various fruit juices and foodstuffs are indicated in Table IV.

TABLE IV

COMPOUND III FROM ALIMENTARY SOURCES

Source	Extraction	μg./liter
Orange juice	pH 1	118
Orange juice	β-Glucuronidase hydrolysis, pH 4.5	116
Orange juice	Original pH	126
Grapefruit juice	Original pH	1500–3500
Lemon juice	Original pH	9.4
Cantaloupe juice	Original pH	None
Watermelon juice	Original pH	None
Beef muscle	β-Glucuronidase hydrolysis, pH 4.5	None
Sustagen (500 g.)	—	None

The presence of compound III in fruits brings up the question of its origin in urines. DeCourcy *et al.* (9) claim that this compound is present mostly in female urines and its presence in urine is due entirely to the ingestion of citrous fruits. These findings could not be substantiated in this laboratory. We did not find any apparent sex difference in the urinary excretion of compound III. In our first experiments on the effect of high potassium intake on the urinary excretion of this compound, the potassium administered as a mixture of citrate, acetate, and bicarbonate* was given four times a day in 100 cc. of orange juice to make it more palatable. This amount of orange juice is grossly inadequate to explain the marked increase of compound III in urine to 2–4 mg./day.

To decide whether the increased urinary excretion of compound III after high K intake is due to the citrous juice or to the potassium itself, the following

*Kindly supplied as Potassium Triplex by Dr. Kenneth Kohlstead, Lilly Co., Indianapolis.

experiments were performed. Two patients were maintained on special diets: the first received the ordinary ward diet not containing any fruit juices and supplemented by 225 meq. of potassium per day, and the second received as only food, a preparation consisting wholly of milk solids, minerals, and vitamins (Sustagen,† Mead, Johnson). The urinary excretion of compound III after 4 and 8 days on the two above diets was in the first patient 700 μg. per day and on the second patient 50 μg. per day. These data, in addition to the effect of high potassium intake, bring evidence in favor of a mixed endogenous and exogenous origin of this compound.

Conclusion

A substance separated from the aldosterone–cortisone zone obtained from the chromatographic purification of urinary extracts has been isolated from urine. It appears similar in all respects to a compound present in citrous juices. Some of its chemical and physicochemical properties are described but do not allow the final elucidation of its chemical structure. This compound in urine is released from its conjugate by incubation with an animal β-glucuronidase preparation. Its chief interest resides in its paper chromatographic behavior which is very similar to that of aldosterone in most systems, in its occurrence in fruit juices, and in its markedly increased excretion in patients receiving a diet rich in potassium and without any citrous juices.

Acknowledgments

It is a pleasure to acknowledge the invaluable help and collaboration of Dr. Lyman C. Craig, Rockefeller Institute for Medical Research, New York; Dr. Norman Brink and Dr. E. Ham, Merck Research Laboratory, Rahway; Drs. Victor Drill, C. M. Kagawa, P. S. Cammarata, and R. Dillon, Searle & Company, Chicago; Drs. J. P. Rosselet and K. M. Mann, Upjohn Company, Kalamazoo; Drs. Julien-Marc Aurèle, André Barbeau, Gilles Leboeuf, Joffre Brouillet, and Barna Vityé of this department.

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†Kindly supplied by Mr. David Menzies, President, Mead, Johnson Co. of Canada. Extraction of 500 g. of Sustagen preparation and its chromatographic purification according to the procedures outlined do not reveal the presence of compound III.

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Contents